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(54) Title: NON-DENDRITIC BACKBONE PEPTIDE CARRIER

(57) Abstract

The present invention relates to a non-dendritic peptide designed for use as a carrier of an immunogenic substance and/or an immune mediator, a construct of said carrier carrying an immunogenic substance and/or an immune mediator, a process for the preparation of immunogens with high and predictable immunogenicity which comprise said non-dendritic peptide carrier, use of such immunogens for the production of vaccines and vaccines comprising an immunogenic substance and/or an immune mediator on the peptide carrier. The invention also relates to diagnostic or therapeutic embodiments using the non-dendritic peptide carrier, to diagnostic or therapeutic compositions and to methods for the use thereof in diagnosis of diseases and pregnancy as well as in therapy. The non-dendritic peptide carrier according to the invention comprises 10-50 amino acids capable of forming a secondary structure in a benign buffer after liberation from the solid phase.

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NON-DENDRITIC BACKBONE PEPTIDE CARRIER

The present invention relates to a non-dendritic peptide designed for use as a carrier of an immunogenic substance and/or an immune mediator, a construct of said carrier carrying an immunogenic substance and/or an immune mediator, a process for the preparation of immunogens with high and predictable immunogenicity which comprises said non-dendritic peptide carrier, use of such immunogens for the production of vaccines and vaccines comprising an immunogenic substance and/or an immune mediator on the peptide carrier. The invention also relates to diagnostic or therapeutic embodiments using the non-dendritic peptide carrier to diagnostic or therapeutic compositions and to methods for the use thereof in diagnosis of diseases and pregnancy as well as in therapy.

The non-dendritic peptide carrier is coupled by its C-terminus through a specifically cleavable "linker" to a solid phase forming a solid phase complex for the preparation of the carrier peptide synthesizing or coupling the immunogenic substance thereon before the non-dendritic peptide carrier is released from the solid phase.

BACKGROUND

In WO 95/31480, an antiparallel "coiled-coil" heterodimer has been used as an immunogen carrier. By use of heterodimers, the preparation of a composition comprising different immunogenic target molecules substances is controlled. The preparation involves mixing two different monomers on which the desired two different target molecules are attached, one on each type of monomer, respectively. As each type of monomer is designed not to form homodimers, only heterodimer formation is favoured. The heterodimers obtained comprise both of the desired two different immunogenic target molecules, one on each monomer. Accordingly, the immunogen composition comprises different immunogen molecules which are coupled on the peptide carrier by conventional coupling

methods and only one immunogenic substance on each monomer is shown. This self-assembling system thus assures the combination of two different substances in the same dimer, e.g. a B-cell- and a T-cell-epitope, respectively. Conformational stabilization was not investigated and the problem of dissociation at low concentrations was not addressed. Subunit peptides are between 21 and 70 amino acids.

In WO 95/11998, structured peptide libraries are shown which are based on the antigenic variations in various pathogenic microorganisms or on a repertoire of T-cell stimulating sequences. The aim is to achieve a broad reactivity or immunogenicity both with respect to the variants of the microorganism and with respect to the host population. The library is synthesized simultaneously in a single peptide synthesis comprising adding a mixture of reactants representing a distribution corresponding to the desired distribution of the final peptide population representing the library. The library is synthesized using solid phase, however, no specific backbone structure is disclosed apart from a conventional lysine containing derivatizable polymer.

In WO 94/29332, an antiparallel intramolecular "coiled-coil" monomer has been used as an immunogen carrier in which the immunogenic substance in the form of peptide sequences are interposed in the linear peptide forming the monomer whereby the total immunogen composition constitutes a pure peptide. Accordingly, a carrier is described which is stabilized by an intramolecular, antiparallel arrangement of two amphipathic α-helices combined by a turn, the stability of which is not concentration-dependent as is the case with α-helix bundles. The structure is not a scaffold designed for carrying multiple immunogenic substances but rather a template for a stable design. The whole peptide can be between 32 and >200 residues. Branch-peptide attachment is not mentioned. No immunization examples are presented.

Cyclic or constrained scaffolds used for attachment of peptides to generate "template assisted synthetic proteins" ("TASP"s) are known (Tuchscherer 1993). These scaffolds were specifically designed to position the attached peptides on a rigid template to enhance the conformational traits of these peptides and has been convincingly shown to do just that by circular dichroism measurements, at least with α-helical peptides. Immunization with TASPs in the presence of adjuvants seems to generate protein cross-reacting antibodies without further conjugation to other carriers. Their synthesis involves a solid-phase synthesis of the scaffold, followed by partial deprotection and synthesis of the attached peptides, followed by cleavage and most often cyclization and extensive purification by HPLC.

Also chemoselective coupling of pre-synthesized peptide thioesters to a bromoacetylated scaffold has been described (WO 95/04543). Different types of structures can be coupled by using orthogonal chemistry (Tuchscherer 1993). Structures, including mixed structures with T-cell and B-cell peptides in various ratios and their use as immunogens have also been reported (Kaumaya 1993)). The scaffold therein consists of two short ß-strands connected by a turn and immunizations were performed with Freund's complete adjuvant leading to antibodies reacting with the peptides and the corresponding native protein. No direct evidence is presented as to the support of structure in the attached peptides, and immunizations are generally performed in the presence of adjuvants.

GB 2 282 813 A describes a scaffold which is cyclised by a thioether linkage and contains lysine residues providing amino groups as attachment points, whereupon cyclic B-cell epitopes as well as linear peptide T-cell-stimulating antigens can be coupled. The scaffold itself is synthesized, cyclised, and purified, and, subsequently, purified peptide antigens, specifically cyclized peptide B-cell antigens and linear T-cell antigens are coupled by chemical means. Also carbohydrates can be coupled and groups with adjuvant activ-

ity, e.g. tripalmitate-Cys-structures can be included. The products are not readily soluble and are administered with liposomes or emulsified for immunizations. There is no data concerning structure support nor is anything presented with respect to ability to induce protein-cross-reacting antibodies. There is no mentioning of synthesis on solid-phase bound scaffold.

In US Patent No. 5229490, synthetic peptide scaffolds are disclosed having a "dendritic" construction. Multiple antigen peptide systems are described in which a large number of antigens are bound to the functional groups of a dendritic core molecule also called "multiple antigenic peptides" ("MAPs"). The antigen-presenting dendrimer is generated by chemical synthesis of a number of "layers" on both amino-groups of α- and ϵ-unprotected lysine, conjugated by its C-terminal to a solid phase, yielding, typically 4 or 8 derivatizable amino groups per one residue of solid-phase-attached lysine. These groups may be derivatized to present other functionalities and/or spacer-moieties (see WO 92/18528 and Tam 1995). By attaching antigenic peptides to the outer amino groups of the solid-phase bound MAP-core, followed by cleavage of the completed structure, a so called high-density presentation is achieved with a minimal "core" carrier structure, each lysine moiety in the outer layer carrying two antigenic peptides. Such MAPs may be highly immunogenic, at least when injected in the presence of adjuvants like Freund's incomplete adjuvant and aluminium hydroxide yielding antibodies exclusively directed against the antigenic peptide "branches". In some cases, however, these antibodies, even if having high affinity to the MAP-bound antigenic peptides, are not very reactive with the native structure from which the peptide is derived (see e.g. Briand 1992). In addition, indications prevail that the number of "branch"-peptides influences the structure of the individual branches (see e.g. Francis 1991), and no facts are presented as to whether the desired retention of the peptide structure in the antigenic peptide branches is present. Their chemical synthesis is

tedious, as the closely situated branch-peptides tend to aggregate. Therefore, protocols generally recommend an "indirect approach", in which the peptide antigens are coupled as pre-synthesized entities (Tam 1993). However, all the problems of no relation to the synthesis are still present.

MAPs have also been found to be difficult to characterize and purify by high pressure liquid chromatography (HPLC). Accordingly, MAPs are normally used for immunization in an essentially unpurified state (apart from e.g. a desalting step or a crude gel filtration step) (see e.g. Tam 1993), deflating the advantage of using a chemical method to produce the conjugate.

In order to improve vaccines and immunizations, an important goal is to refine the antigen used and especially to ensure its purity and its chemical definability while still retaining its ability to evoke a beneficial immunological response. However, the known carrier peptides and production of such peptides and immunogenic compounds are inadequate in this respect. Generally, liposome-formulation or formulation with oil-in-water adjuvants is needed to create an adequate antibody response.

Well-established methods for increasing the immunogenicity of peptides exist (see e.g. Plaue 1990). Conventional methods generally involve the conjugation of the peptide by chemical means to a carrier molecule (see e.g. van Regenmortel 1988). The chemical conjugation methods, well-known to those skilled in the art, comprise coupling with glutaric aldehyde (coupling to amino- and thiol groups), carbodiimides (coupling to amino groups), m-maleimide benzoyl-N-hydroxysuccinimide esters (coupling from amino- to thiol-groups), and coupling carbohydrates through oxidation followed by reaction with primary amino groups and reduction as well as coupling thiols by specific reagents (see e.g. van Regenmortel 1988). The carrier is typically a naturally-derived protein, as e.g.

albumin, ovalbumin, purified protein derivative (PPD) from *M. tuberculosis*, keyhole limpet hemocyanin, avidin, diphtheria toxoid, tetanus toxoid, etc.

The immunization schedule often and typically entails multiple sequential injections of the peptide-carrier conjugate intramuscularly, subcutaneously or intradermally together with adjuvants including Freund's complete adjuvant, Freund's incomplete adjuvant, Emulsigen^R, Titermax^R, aluminium hydroxide, etc. The only adjuvant at the present date allowed for human use is aluminium hydroxide. A rather new type of adjuvant is the immunostimulating complex ("Iscom") (Morein 1984) in which a combination of amphiphilic antigen, quil A, cholesterol, lipid and phosphatidylcholine leads to the formation of small (35 nm in diameter), highly immunogenic particles in which the antigen is embedded in the membrane turning its hydrophilic parts outwards. This method has especially shown to work with viral antigens. Iscoms may be used as a peptide carrier/adjuvant by conjugating the peptides by chemical methods to a preformed Iscom containing a suitable protein (Larsson 1993). Another type of carrier functioning as an adjuvant and usable with synthetic peptides is liposomes containing lipid A and a reactive group for chemical coupling of peptides (Friede 1993).

Methods for producing immunogenic peptides by carrier-coupling, however, are largely empirical, and, normally, it is not possible to predict the optimal carrier, conjugation method, and conjugation density for the induction of a desired immune response. Important parameters such as the orientation by which the peptide antigen is coupled to the carrier are not controlled, although of major importance for the produced antibodies. Accordingly, the problems, which are overcome according to the present invention, include problems of chemical definability as follows:

1. The ratio of peptide to carrier is only measured with some difficulty, e.g. by amino acid analysis, radioactivity count-

ing or by estimates based on accurate molecular weight determinations.

2. With most chemistries, the peptide is coupled to the carrier in random orientation and by the attachment of a varying number of functional groups along the peptide chain possibly leading to the destruction of the charge-distribution and structure of the peptide.

3. Peptide-peptide- and carrier-protein - carrier-protein polymer-formation can only be excluded by careful case-to-case optimization of reaction conditions possibly in combination with post-reaction clean-up, e.g. by size-exclusion chromatography.

4. Naturally derived carrier proteins are not chemically totally characterized.

5. The conjugation procedure increases the time needed to produce the peptide-immunogens, adding a post-synthesis step, and also increasing loss of material.

6. Antibodies formed against the carrier protein and the conjugation group may cause problems.

7. Naturally derived carrier proteins of "immunization" quality are quite expensive.

Finally, as a rule, naturally derived (or recombinant) carrier-proteins are not allowed for administration in humans as immunogens.

According to the present invention, the drawbacks mentioned above can be circumvented by using totally synthetic immunogens, e.g. synthetic peptides coupled to a synthetic peptide carrier, provided that this carrier presents the peptide immunogen at high density, supplies the necessary T-cell-epitopes, and supports the conformation of the peptide.

Benefits include the possibility of direct, sequential synthesis on a solid-phase bound peptide carrier (no need for post-synthesis operations), and a greater variety of chemical methods being amenable, e.g. allowing different substances to be coupled by orthogonal chemistries and allowing control of orientation of the peptide. Also, the development of antibodies to carrier proteins and coupling groups is avoided.

The chemical synthesis of peptides by solid-phase synthesis is well known (Merrifield 1963, Atherton 1978). Peptides are synthesized sequentially from the C-terminus, which is bound to an insoluble solid phase by a specifically cleavable chemical "linker". After completion of the synthesis, the peptide is liberated by cleavage of the linker. Four groups of chemistries are combined, the first one being used for activating the α-carboxylic group of the amino acids to be coupled, the two other ones being used for protecting α-amino groups and side-chain groups, respectively, and the fourth one controlling the lability of the peptide-to-solid phase linker.

In one embodiment, the present invention relates to a copolymer consisting of a conventional solid-phase peptide synthesis polymer to which is coupled an oligopeptide containing a number of freely accessible functional groups on which additional peptides can be synthesized or coupled or other entities can be coupled. Peptide synthesis on the copolymer can be performed by conventional methods of solid-phase synthesis. After synthesis or coupling, the whole complex can be cleaved and brought into solution and can be purified and analysed by conventional means. Additionally, the coupled peptides can be sequenced. The complex presents the coupled peptides very efficiently, increasing the immunogenicity of the coupled peptides considerably. The complex can thus be used as an immunization means, either alone or in combination with Iscom-forming agents or with other adjuvants.

For a full chemical definition, it is preferable, if possible, to synthesize the appropiate antigen chemically, rather than to derive the antigen from natural sources. To be feasible, such a synthesis should be economical and lead to practical amounts of the antigen with the desired purity. Such easily and economically synthezisable antigens include short peptides, i.e. peptides comprising fewer than approximately 30 amino acid residues.

The use of short synthetic peptides as antigens in vaccines has a number of advantages, including increased safety, increased reproducibility, low production costs, and possibility of choosing non-mutating epitopes (see e.g. Shinnick 1983). Antibodies generated against synthetic peptides are directed against predetermined parts of a protein; this is not possible by other methods. Antibodies may also be directed against non-immunogenic parts of proteins or against non-peptide antigens, e.g. carbohydrates or haptens. Cancer, viral, including HIV, bacterial, and parasitic infections have been investigated with respect to peptide-based vaccines.

In a basic research setting, peptides can be used for the production of antibodies against non-identified proteins of which the gene sequence is known and for mapping sites in a protein. In the vaccine field, totally synthetic vaccines are advantageous for several reasons including economy, high batch-to-batch reproducibility, increased stability, exclusion of impurities, and the above-mentioned possibility of obtaining a "clean" response only directed against the critical part(s) of the pathogen. Such synthetic construct also have many application as antigens in diagnostic assays and as therapeutics.

A neutralizing response against a whole microorganism raised by a single short synthetic peptide has, however, been demonstrated in only a few special cases. This is due to the fact that peptides, as a rule, are not immunogenic, i.e. not able

to induce a substantial immune response by themselves. Short peptides are not believed to be big enough to ensure a full immune response (humoral as well as cellular response) because the major histocompatibility complex does not bind peptides which do not have the right sequence and which lacks a synergistic or cooperative effect seen with bigger immunogens presenting several epitopes to the host cell at the same time (multivalency). If antibodies are obtained, they typically have low affinities, as the conformation of short peptides is not defined very well in aqueous environments; therefore, the resulting antibody is not selected to the constricted conformer of the peptide in its polypeptide-enclosed, natural state. This leads to an undesirable low degree of cross-reactivity between an anti-peptide antibody and the corresponding protein detrimental to most vaccine-applications where the antibody-response should neutralise an invading pathogen, e.g. by binding to a specific toxin, or by binding to surface-molecules implicated in binding of the pathogen to host tissue or by binding to a pathogen-specific antigen preparing the microorganism for phagocytosis.

The design of short peptides (< ca. 30 amino acids) which retain structure in aqueous environment is a major task. The structure of short peptides may be increased by chemical derivatizations restricting the conformational freedom of the peptide, e.g. by cyclization or polymerization (see e.g Robey 1992 and Gilon 1991) or by the inclusion in the chain of structure-nucleating substances, eg. turn- or α-helix-inducers (e.g. Kahn 1993, Unson 1984, Kemp 1990, Hinds 1991, Días 1993 and Bambino 1994) or chelated metal-ions (e.g. Regan 1995). However, the only single unmodified peptide structure capable of retaining conformation in water is the amphipathic α-helix (Mant 1993) which is stabilized by aggregation into homo- or heterodimers or oligomers. An amphipathic helix has a hydrophobic side occupying one half face of the helix along its axis and a hydrophilic side on the other half. Amphipatic α-helices typically combine in "bundles" shielding the hydrophobic face and exposing the

hydrophilic side to form parallel or antiparallel homodimers (Zhu 1993) or heterodimers and oligomers (Zhu 1992).

In the present context, the definitions below are used:
A peptide carrier is a substance comprising a peptide that serves as a carrier of one or more moieties which may themselves be peptides, each moiety being chemically coupled to said carrier peptide through a linkage involving an amino acid side chain of the carrier peptide.

A non-dendritic peptide carrier is a peptide carrier that does not contain any double-derivatizable building blocks which are substituted in any of the derivatization groups with another similar or different double-derivatizable building block, said non-dendritic carrier peptide further providing at least two derivatizable functional groups.

In the description of the non-dendritic peptide carriers and the derivatized non-dendritic peptide carriers, the following terms are used:

Backbone peptide denotes the non-dendritic peptide carrier itself. Branch-peptide or "branch-moiety" denotes the peptide or moiety coupled to the non-dendritic peptide carrier which thereby is derivatized. Attachment point denotes the functional groups in the non-dendritic peptide carrier available for derivatization.

A lipidic moiety is defined as an alkyl or alkenyl fatty acid bound by its carboxylic function as an amide or an ester.

A solid-phase linker for peptide synthesis is a molecule with at least two functional groups, one used for providing a stable covalent linkage to the solid phase polymer, and the other one used for attaching the C-terminal amino acid by a stable covalent linkage during peptide synthesis, said amino acid attaching linkage being stable to the peptide synthesis conditions but having a defined lability to specific chemical

treatments by which the produced peptide can be liberated either as a free acid or a C-terminally modified peptide, depending on the chemical treatment.

An antigen is a substance which is reactive with a specific antibody or T-cell.

An immunogen is a substance capable of inducing an antigenic response including antibodies and a T-cell response.

An immune mediator is a substance which is capable of regulating the activity of the immune system. Such a regulating effect may refer to an activation of the immune system as well as a down regulation of the immune system. The term immunomodulator is used interchangeably with the term immune mediator.

An adhesion molecule is derived from a cell surface and is capable of binding to another specific cell surface derived molecule. Selectins and CAM molecules are examples of adhesion molecules.

A T-cell stimulatory peptide, -antigen, -protein and a T-cell antigen are used interchangeably and denote a molecule that is capable of stimulating the proliferation of a specific T-lymphocyte clone in the immunized host resulting in a T-lymphocyte-mediated immunogenic response, as well as a molecule that is reactive with a specific T-lymphocyte clone.

A B-cell antigen is a molecule that is reactive with a specific B-lymphocyte clone or that elicits a B-lymphocyte-mediated immunogenic response in a subject or test animal.

A benign buffer is a physiologically compatible aqueous buffer with a pH between about 6 and about 8 and a salt concentration between about 50 mM and about 500 mM, preferably between about 100 mM and about 200 mM.

Peptide and polypeptide are used interchangeably to denote a polyamide chain of amino acids from 2 amino acids to 100 or more amino acids long.

A stabilized or supported secondary structure is defined as a preferred conformational state of the peptide in question, obtained by restricting the rotation of the peptide bonds comprising the peptide into a relatively fixed and well-defined structure by a "secondary structure supporting moiety".

Peptide amino acid residues are always counted from the N-terminus, the N-terminus being number 1.

Sequences for peptides and polypeptides are given in the order from the amino terminus to the carboxyl terminus.

All amino acids are represented by standard one- or three-letter abbreviations as commonly used in the art.

As used herein, natural amino acids are the 20 amino acids, either in L- or D-forms commonly found in proteins, e.g. alanine, aspartic acid, asparagine, arginine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryphtophan, and alanine.

Unnatural amino acids include, but are not limited to, D- and L-forms of epsilon-aminohexanoic acid, gamma-amino butyric acid, alpha-aminobutyric acid, alpha-aminoisobutyric acid, alpha-aminoadipic acid, allo-threonine, allo-isoleucine, 7-aminoheptanoic acid, norleucine (2-aminohexanoic acid), norvaline (2-aminopentanoic acid), delta-aminovaleric acid (5-aminopentanoic acid), 11-amino-undecanoic acid, t-Butyl-alanine, t-Butyl-glycine, gamma-carboxyglutamic acid, citrulline, homocysteine, homocitrulline, homoarginine, homophenylalanine, delta-hydroxylysine, 4-hydroxy-proline,

isoasparagine, isoglutamine, isoserine, penicillamine, phenylglycine, thyroxine, ornithine, and the like.
The term amino acid denotes both groups.

A PNA or peptide nucleic acid substance is a molecule consisting of a peptide chain of unnatural amino acids selected from amino acids containing naturally occurring DNA- or nucleobases in their side chains (see WO 95/01369).

DESCRIPTION OF THE INVENTION

It is an object of the present invention to provide a general non-dendritic peptide carrier that provide a high-density presentation of bioactive moieties in a predefined particular arrangement, orientation and stoichiometry. The core peptide carrier is not immunogenic and the construct is chemically unambigous. Coupled antigens are presented very efficiently, increasing their immunogenicity considerably. The complex can thus be used as an immunization means, either alone or in combination with Iscom-forming agents or with other adjuvants.

The defined oligopeptide (the non-dendritic peptide carrier) of the present invention comprises more than one accessible functional group and is furthermore derivatized in one or more locations by a strongly hydrophobic moiety, preferably a long-chain fatty acid like palmitic or myristic acid, and bound through its C-terminal amino acid to a polymer called a solid phase. On said oligopeptide, antigenic entities can be coupled by chemical methods by direct synthesis or by coupling "en bloc", leading to a branched complex that can be cleaved in its intact entity from the solid phase and can be characterized and if necessary purified therefrom by standard methods.

The invention further comprises a method for the synthesis of the oligopeptide as well as the synthesis of branched complexes.

The non-dendritic peptide carrier can be synthesized by standard Fmoc-chemistry using active esters or in situ/preactivated amino acids (e.g. TBTU/HOBt/NMM in NMP) amino acid active esters or symmetric anhydrides (see e.g. Atherton 1989) on a suitable solid phase which include commercially available polymers as Polyhipe, Wang, Novasyn as well as any other polymers that can be derivatized and are insoluble in the solvents used for synthesis. Depending on the type of solid-phase polymer, the substitution should be between 0.01 and 0.1, preferably 0.05 to 0.1 and even more preferably about 0.05 mequivalents pr. gram solid phase; Novasyn is typically used at about 0.05 mequivalents pr. gram. The commercially available Novasyn KB resin, which contains the HMB-linker (4-hydorxy-methyl benzoic acid), hydroxyl groups at around 0.150 mequivalents pr. gram are available for peptide coupling. A substitution of 0.05 mequivalents pr. gram is then achieved by performing the esterification of the C-terminal amino acid residue of the peptide with a short incubation time, resulting in partial substitution only of the hydroxyl groups of the linker. Subsequently, and before alpha-amino deprotection of the coupled amino acid, additional free hydroxyl groups are blocked by incubation with a large surplus of acetic anhydride in the presence of the estrification catalyst N,N-dimethylaminopyridine (DMAP). After peptide synthesis, HMB can be cleaved to form either the free acid, the carboxamide, the acylhydrazine, etc. The linkage of the peptide to the solid-phase can be, but is not limited to be, constituted by base-cleavable linkers. Any other scheme employing orthogonal or graded protection of side-chains, protection of α-amino-groups and stability of the linker is amenable. In a preferable method lysine residues are orthogonally protected in their epsilon-amino groups as e.g. Fmoc-K(Mtt)-OH, Fmoc-K(Dde)-OH and Fmoc-K(Aloc)-OH, in which side chain protecting groups are removable by 1% TFA, 2% hydrazine and Pd(0)(catalytic hydrogenation), respectively. After synthesis, alpha-amino lipid-coupling and selective lysine-side chain demasking, synthesis or coupling of branch peptides or moieties can

then be accomplished with no risk of interference from unmasked functional groups in the side chains of other amino acids in the backbone peptide, especially the carboxylic acid deriving from E. If used unprotected, this side chain carboxylic group may lead to formation of lactams with any free amino group generated during branch peptide synthesis or coupling or with the amino groups of the backbone lysine residues themselves, due to the generation of or presence of carboxylic activating species during branch peptide synthesis or coupling.

Also preferred is a backbone peptide carrier containing two types of protection for lysine, a selectivily cleavable one for attachment point lysines as well as Boc-protected lysines at the "c"-non-attachment positions.

Especially preferred is a solid-phase backbone peptide complex, in which a selectively protected subgroup of lysine residues constitute attachment points after deprotection, and in which all other protection groups as well as the solid-phase linker are cleaved by the same chemical treatment, preferably 95% TFA with appropiate scavengers.

Other preferred embodiments of this type include Boc-chemistry with benzyl ester side-chain protected amino acids, the peptide being linked to the solid phase through base-labile linkers such as HMB, 3-nitro-4-(2-hydroxyethyl)benzoic acid (NPE), 4-nitrobenzophenone oxime or photo-cleavable ones like the acid-stable 2-bromopropionyl-α methylphenacyl ester, Fmoc-chemistry (tBu and Boc side-chain protection) in combination with typical Boc-linkers (cleavable by HF or TFMSA only) such as substituted benzyl esters (chloromethylphenyl or 4-hydroxymethylphenylacetic acid (PAM)), benzhydrylamine derivatives (e.g. 4-methylbenzhydrylamine) leading to peptide carboxamides on HF-cleavage or photocleavables like the α-bromo-phenacyl type mentioned above, 3-nitro-4-hydroxymethylbenzoic acid (ONb), and 3-nitro-4-aminomethylbenzoic acid (Nonb, leading to the peptide carboxamide). Both strategies

may also be used with hydroxy-crotonyl type linkers cleavable by catalytic hydrogenation by Pd(0) in the presence of a weak nucleophile (see e.g. Atherton 1989). A group of particularly useful linkers are water-cleavable moieties such as glycolic acid derivatives (Hoffmann 1994).

In one embodiment, the present invention comprises a non-dendritic lipopeptide carrier ("backbone"), preferably consisting of between 10 and 50 amino acids with an α-amino-bound fatty acid, preferably palmitic acid or myristic acid or tripalmitate-Cys (see Fig. 4C), and covalently bound by its C-terminal carboxylic function to a suitable solid phase by a specifically cleavable linker.

This type of peptide can preferably be synthesized on the base-cleavable HMB-linker coupled to the solid phase. Acid-labile side-chain protection is used, for lysine (K), Boc (tert-butoxycarbonyl-). After synthesis of the protected solid-phase bound peptide chain, the amino-terminal Fmoc-group is removed by treatment with piperidine, and palmitic acid is coupled as the symmetric anhydride or by TBTU/-HOBt/NMM in NMP until the Kaiser test is negative. Subsequently, lysines are side-chain-deprotected by TFA/water (95%, 1 hour, room temperature) or by TFA/DCM (50%, 6 times 30 minutes, room temperature) and washed extensively before further use or drying and storage. The optimal loading density of the peptide on the solid phase is typically lower than the initial density of functional groups on the solid phase. The loading density is controlled in the esterification step (coupling of the first amino acid to the solid phase) by varying the incubation time; a short incubation time gives a lower loading density. It was found that loading densities in the range 0.05 to 0.1 milliequivalents pr gram solid phase was optimal when using Novasyn resins.

After attaching branch-moieties, the complex can be liberated as a free acid by cleavage with aqueous NaOH. By cleavage with methanolic ammonia, a peptide amide is liberated, the C-

terminal amide being more advantageous with regard to supporting α-helical structures. Cleavage with hydrazine in DMF or NMP leads to the peptide hydrazide.

The lipopeptide backbone contains a number of freely accessible functional groups, preferably amino groups, preferably more than 2, spaced evenly along the peptide-chain. In a variation of this structure, the lipopeptide backbone contains as functional groups carboxylates. In yet another variation, thiols are included as the functional groups. In still another variation carbonyls, haloacetyls, hydrazides, α-oxoacyl, amino oxyacetyl (i.e. hydroxylamine), cysteine, maleimide-groups are derived by chemical methods from primary amino groups. Antigenic entities, or other molecules may be coupled stepwise or en bloc to these backbone-peptide groups and may be peptides/ carbohydrates/haptens.

Peptides are readily amenable to stepwise chemical synthesis on primary side-chain localised amino-groups, preferably the ε-amino group of lysine or the delta-amino group of ornithine. These groups also can function as targets for the chemoselective ligation (see below) of unprotected peptides, carbohydrates, or any other entity containing a suitable functional group. En bloc coupling may be performed by chemical methods well-known to those skilled in the art of conjugating proteins to proteins and peptides to proteins, including methods for temporary protection of side-chain functionalities in the molecule to be coupled. Such preferred coupling methods include: coupling with glutaric aldehyde (coupling to amino- and thiol groups), carbodiimides (coupling to amino groups), m-maleimid benzoyl-N-hydroxysuccinimide ester (coupling from amino- to thiol-groups) carbohydrates through oxidation followed by reaction with primary amino groups and reduction, thiols by the N-succinimidyl 3-(2-pyridyldithio) propionate- (SPDP-) method (see e.g. van Regenmortel 1988).

Preferred temporary protection methods include citraconylation (primary amino group protection, the protecting group being released by low pH) and Fmoc-derivatisation by Fmoc-succinimide (primary amino group protection, the protecting group being released by piperidine). Also side-chain blocked synthetic peptides may be coupled by such methods in addition to coupling by carboxyl-activation in organic solvents.

A preferred group of methods for en bloc couplings is "chemoselective ligation" (reviewed by Tam (1995)) because these methods can be used for coupling of unprotected fragments, especially peptides, and especially synthetic peptides and furthermore allows the coupling-polarity or -direction to be controlled. These methods have been used to couple peptides to peptide scaffolds including MAPs (e.g. Lu 1991) and TASPs (e.g. WO 95/04543).

A typical example of a preferred chemoselective method is the reaction of a thiol nucleophile with an appropiate electrophilic group. One such reaction is the reaction of haloacetyl groups, preferably chloroacetyl and even more preferably bromoacetyl groups with alkylthiols leading to the ready formation of a very stable thioether bond (Wetzel 1990, Robey 1992). Alkylthiol groups may be introduced anywhere in a peptide or another molecule by coupling cysteine, and haloacetylation is readily performed on primary amino groups by reaction with haloacetic acid anhydride (e.g. Robey 1992). It can also be introduced during peptide synthesis using a preformed derivative with the haloacetyl already incorporated as the side-chain (Ivanov 1995). In a preferred embodiment, haloacetyls are introduced on the free ϵ-amino groups on the lipopeptide backbone peptide bound to the solid phase and the peptide or molecule to be attached is modified with cystein at the desired position (e.g. Lu 1991 and Tam 1993). A similar and also preferred reaction is obtained by reacting an acylthiol (thiocarboxylate) group with a haloacetyl group, by which a thioester is formed (Schnölzer 1992). Acylthiol groups can be formed at the C-terminus of the peptide to be

coupled (by commonly known methods of solid-phase peptide synthesis (Yamashiro 1988)); by this method, no cysteines need to be introduced. The thioester is unstable at neutral and basic pH-values (Tam 1995). If, however, the reaction is allowed to take place between a N-terminal cysteine and the acylthiol-group, a spontaneous rearrangement leads to a stable amide bond and reestablishment of the cysteine side-chain thiol (Dawson 1994). This thiol group, if undesirable, can be blocked (alkylated) by methods known in the art. Other selective reactions with thiol groups include reaction with maleimide groups (addition reaction), leading to a stable thioether. Maleimide groups may be introduced by acylating primary amino groups or by coupling as the N-terminal entity during solid-phase peptide synthesis (see Tam 1995).

Also thiol-disulfide exchange reactions leading to the establishment of a new disulfide may be preferred as in the SPDP-coupling scheme (Carlsson 1978). This binding although covalent is, of course, very sensitive to reducing conditions, which can be used for the deliberate release of the molecule. Another preferred example of a chemoselective reaction is the reaction between an aldehyde and a weak base, preferably the reaction of aldehyde with hydrazide (acyl-hydrazine) leading to a relatively stable hydrazone which may be reduced to a stable substituted hydrazine (see e.g. Tam 1995) or the reaction of an aldehyde with hydroxylamine leading to a stable oxime (Rose 1994). These reactions can be used for the selective coupling of reducing carbohydrates or mildly oxidized carbohydrates (monomers, dimers, oligomers, polymers or as part of glycoconjugates oxidised by periodate) through the aldehyde group of the carbohydrate. The reaction of hydroxylamine or hydrazide with periodate-oxidized N-terminal serine, threonine or cysteine (which are particularly sensitive to this oxidation leading to the selective introduction of α-oxo-acyl groups at the N-terminus in a peptide), the preferred amino acid being serine, leads to the chemoselective coupling of a hydroxylamine or hydrazide-containing peptide with the N-terminal of another peptide

(Rose 1994, Gaertner 1992). Acyl-substituted hydrazines (hydrazides) can be introduced in solid-phase peptides bound by the HMB-linker to the solid phase by cleaving the linking ester by hydrazine. Also, acylhydrazine may be introduced, by derivatising a free primary amino group with Boc-monohydrazide succinic acid or with 4-Boc-monohydrazinobenzoic acid. Hydroxylamine is conveniently introduced by reaction with protected aminooxyacetic acid (see Tam 1995). In another method, carbonyl groups are introduced on primary amino groups by protected acetal alkanoic acids (unstable to HF and thus limited to Fmoc-strategies of solid phase peptide synthesis) (see Tam 1995). A special and preferred method entails the reaction between an aldehyde and a N-terminal cysteine to yield a thiazolidine. An especially useful aspect of this method is that it introduces a heterocyclic ring that may provide conformational stability ("rigidity") to the peptide coupled.

From the above-mentioned examples of coupling chemistries it is clear that, with the present invention, antigens may be coupled through a big number of functional groups and that antigens can be coupled in any orientation desired to the non-dendritic backbone peptide; also, it is clear that synthetic (protected or unprotected) as well as naturally-derived peptides can be coupled, as well as other antigens, especially carbohydrates.

The lipopeptide non-dendritic backbone as well as the complete branched complex of the present invention are designed to fold into a highly ordered structure in aqueous environment. In an especially preferred and claimed embodiment studied in Example 1, the amino acid sequence of the oligopeptide is defined by a number of repeated "heptads" conferring a tendency to form amphipathic parallel α-helices (homodimeric coiled coils) to the oligopeptide. This was based on the following design considerations:

As facing residues (a and d positions) I, L, and V are preferred. As top and bottom residues (e and g positions) D and E and K, R, and H, respectively, are preferred. Outside (non-interacting) residues (positions b, c, and f) are avialable for other interactions including e.g. lactam-bridge formation, histidine chelation, and attachment points.
A preference for parallel assembly is generally seen, and when a strong hydrophobic interaction is allowed (e.g. I-L), homodimerization is preferred, even when like-charged e and g pairs are present, but oppositely charged e-g-pairs favours homodimer formation.

Dimeric coiled coils are concentration dependent which is not suitable for in vivo use. An object of the present invention is to exclude or decrease concentration dependence by introducing further stabilising elements, typically lipidic moieties.

The basic heptad was chosen as VAKLEAK. V and L constitute big hydrophobics at the strand-to-strand interacting a and d positions. E and K supply opposite charges at e and g positions, favouring parallel packing. The very high helix propensity residue A is occupying the rest of the positions, except c, which is used for K creating a side-chain attachment point on the outside of the helix.

The basic sequence was modified to the applied sequence:

abcdefgabcdefg
Palm-AVHKLEHKVAKLEAKGKGKY,Palm" signifying α-N-attached palmitic acid.

The peptide has the minimal length required for stable helix-formation (2 turns). HX_3H is introduced at the helix-outside vis-à-vis each other at two separate turns to create a helix-indicating and -stabilising metal-chelating site. The N-terminal charge is blocked by the α-N-attached palmitic acid and the C-terminal charge (that is also counteracting the α-helix macrodipole) is preferably blocked, e.g. as the amide.

A C-terminal GKGKY-sequence is added, however, that serves as an α-helix C-terminal capping sequence blocking the charge. Furthermore, this non-structured stretch can be coupled through its C-terminal residue to other groups without affecting helix-stability. Y is included as a 280 nm absorbing reporter group and K-residues as attachment groups.

In another preferred design, the K-residues at the g-positions are selectively protected, in order to preserve the stabilizing positive charge at the g-positions after the deprotection subsequent to attachment of side-chain branches to the other K-residues.

In yet another preferred embodiment, b-positions are occupied by K-residues too, to allow for even more attachment points on the outside of the helix.

In yet another preferred embodiment, a lactam-bridge, stabilizing the helix is formed between outside-residues at subsequent b and f positions in one of the heptads, preferably the N-terminal one, by substituting the H-residues with a E/K-pair. This is accomplished easily by a skilled operator by employing orthogonal protection of these side-chains and affecting lactam-formation by BOP/DIEA or TBTU/HOBt/NMM or analoguous activation methods in situ with the peptide still attached to the solid phase.

The structure of this complex leads to a high-density presentation of attached antigenic entities to the surroundings, either by interaction with hydrophobic surfaces by the lipidic part of the structure or further enhanced by self-aggregation into micellar structures, in both cases retaining the dimer-structure, even at low concentration.

Moreover, the close packing of attached peptidic antigens allows neighbour-strand interactions that support inherent conformational traits in the peptides, thereby increasing the similarity of the peptide conformation with the conformation

of the same peptide as part of a polypeptide sequence, a major concern in the art of mimicking natural epitopes by short synthetic peptides. Additional peptides ("auxiliary" peptides) may be introduced, e.g. T-cell stimulating peptides.

The structure thus represents a totally synthetic, fully chemically defined immunogen. The peptide carrier part (the lipopeptide backbone) itself is not immunogenic when derivatized. It is characteristic of the lipopeptide backbone that it can be synthesized by standard solid-phase chemical methods and that antigens, peptides, carbohydrates or haptens and naturally-derived or synthetic molecules may be coupled to the premade solid-phase bound lipopeptide by known methods and that peptides may be synthesized directly on the solid-phase-bound structure by known solid-phase peptide synthesis methods. Following liberation from the solid phase the whole complex form stable aggregates that can be analysed by HPLC and used for immunization. Attached peptides are sequencable without interference from the oligopeptide lipopeptide. When formulated together with Iscom-forming substances, the complex is inserted into the Iscom-membrane by its lipid part, presenting the branched structures on the outside.

In a specific variant of the solid-phase chemistry, the simultaneous synthesis, but separate release of both peptide-polymer-bound as well as free peptide can be accomplished. It is often useful to be able to test the reactivity of the antibodies obtained after immunization with the free peptide. The lipopeptide-structure in another preferred embodiment is designed to present a free amino-terminus. This is done by coupling the lipidic part of the molecule to a side-chain of an internal amino acid. This lipid-coupled side-chain is preferably situated at or near one of the termini of the lipopeptide.

In yet another configuration, the basic structure is combined with additional (auxiliary) peptides (T-cell epitopes, tuft-

sin or other immunomodulating substances), either as an integral part of the lipopeptide or a part of the coupled antigens.

In a variant of the invention, the structure is designed to incorporate the peptidic antigen as a loop in a linear sequence, creating loop-mimetics that will also incorporate into lipohilic membranes.

In yet another embodiment, the structure simply functions as an anchor attaching carbohydrates to lipohilic membranes. In this version, the peptide is synthesized as the hydrazide, the hydrazide being highly reactive with carbonyl groups on gently oxidised carbohydrate, and the peptide sequence incorporating T-cell epitopes, tuftsin or other immunomodulating auxiliary peptides. The peptide hydrazide is obtained by cleaving the 4-hydroxymethylbenzoic acid linker with hydrazine.

In yet another embodiment of the structure, a specific "peptide nucleic acid" ("PNA") sequence or a DNA-intercalating substance may be included with the purpose of binding a specific piece of DNA by hybridization.

The invention, in addition to the use of using such constructs for immunization with the aim of inducing antibodies against the antigenic branches, also comprises using such constructs as therapeutics as well as using such constructs as diagnostics.

The invention also relates to diagnostic embodiments using the peptide of the solid phase complex according to the invention, to diagnostic compositions containing said peptide, and to methods for the use thereof in diagnosis of diseases and pregnancy. The invention further relates to vaccines in which a vaccine component comprising the peptide of the solid phase complex to which an immunogenic agent is

linked and to methods of immunizing animals and conferring resistance against diseases using said vaccine component.

Another important aspect of the invention relates to a therapeutic component in which a therapeutic agent is linked to the peptide of the solid phase complex, to therapeutic compositions containing the therapeutic component and the use use thereof for treatment and/or prevention of diseases or for regulations of the immune response.

In clinical diagnostic embodiments of the invention, a diagnostic agent linked to the peptide of the solid phase complex forming a diagnostic component of the inventions may be used in combination with appropriate means, such as a label, to determine the presence of a specific molecule via its binding to the diagnostic component. In particular, the invention relates to the diagnosis of infectious diseases derived from bacteria, vira, and parasites as well as detection of cancerous diseases, malignant tumours, and autoimmune diseases. Furthermore, the invention can be used in the detection of pregnancy by linking molecules capable of binding to molecules indicative of or derived from pregnancy to the peptide of the invention, and this aspect therefore constitutes another interesting part of the invention.

The diagnostic agent to be linked to a peptide of the solid phase complex of the invention may be any molecule. It may be naturally derived or chemically synthezised. A particular interesting aspect of the invention is the linking of a polypeptide, a carbohydrate, a lipid, and any glycosylated or lipidated form thereof or a nucleotide sequence which is capable of binding to molecules derived from or indicative of pregancy or a disease, including cancerous diseases, autoimmune diseases and infectious diseases.

Typical methods of detection might utilize, e.g., radioactive species, enzyme-active or other marker ligands such as avidin/biotin and hapten/anti-hapten detection systems, which

are detectable directly or indirectly. In preferred diagnostic embodiments, one will likely desire to employ an enzyme tag such as alkaline phosphatase or peroxidase rather than radioactive or other reagents that may have undesirable environmental effects. Enzyme tags, e.g., often utilize colorimetric indicator substrates that are readily detectable spectrophotometrically, many in the visible wavelength range. Luminescent substrates could also be used for increased sensitivity.

One particular interesting embodiment of the invention is the use of an antigen as the diagnostic agent. In both immunodiagnostics and vaccine preparation, it is often possible and indeed more practical to prepare antigens from segments of a known immunogenic polypeptide or carbohydrate. Certain epitopic regions may be used to produce responses similar to those produced by the entire antigenic polypeptide or carbohydrate. Potential antigenic or immunogenic regions may be identified by any of a number of approaches, e.g., Kyte-Doolittle antigenicity analyses (see, e.g., Kyte and Doolittle, 1982; or U.S. Patent No. 4,554,101). Hydrophobicity analysis assigns average hydrophobicity values to each amino acid residue and from these values average hydrophilicities can be calculated and regions of greatest hydrophilicity determined.

Preferred immunoassays are contemplated as including various types of enzyme linked immunoassays (ELISAS), immunoblot techniques, and the like, known in the art. However, it is readily appreciated that utility is not limited to such assays, and useful embodiments include radioimmunoassays (RIAs) and other nonenzyme linked antibody binding assays or procedures. Other immunodiagnostic embodiments of the invention may be based on immunoprecipitation assays, agglutination assays or the like.

Methods of diagnosing pregnancy or disease are also included in the invention. In one embodiment, an antibody-based method

includes obtaining a sample from a patient suspected of having the disease or being pregnant, exposing that sample to the diagnostic component of the invention to which one or more epitopes of a polypeptide, carbohydrate or the like, derived from or indicative of the disease to be diagnosed are linked and finally determining a reactivity of the antibody that may be in the sample with one or more such epitopes. The substantial immunological reactivity measured is indicative of the presence or absence of the disease. Typical samples obtainable from a patient include human serum, plasma, whole blood, cerebrospinal fluid, seminal or vaginal fluids, exudates, and the like.

Several variations of antigen-based methods are contemplated for development; e.g., an indirect ELISA using one or several epitopes attached to the peptide of the invention alone or in various combinations as antigen(s). Optimal concentration of the antigen could be determined by checker board titration and diagnostic potential of the epitope or whole molecule, such as a polypeptide or carbohydrate, from which the epitope is derived. The assay may be further examined or improved by testing serum from e.g. an experimental animal at different stages of a disease. These results could indicate the relative time course for sera conversion for each of the assays.

In further aspects, the present invention concerns a kit for the detection of a molecule, including a polypeptide, a carbohydrate or a nucleotide sequence capable of binding to the specific selected diagnostic agent linked to a peptide of the invention forming the diagnostic component of the invention, together with means for detecting a specific binding between the diagnostic component and the molecule capable of binding thereto. Examples of suitable means include labels attached directly to the diagnostic component, or a secondary antibody having specificity for the diagnostic component. Alternatively, avidin-biotin mediated *Staphylococcus aureus* binding could be used. For example, the monoclonal antibody

may be biotinylated so as to react with avidin complexed with an enzyme or fluorescent compound.

A particular embodiment of the invention concerns kits for detection of antibodies directed against an antigen linked to the peptide of the solid phase complex. The antigen for the kit(s) may be a polypeptide, a carbohydrate, a lipid or a part thereof or a nucletide sequence obtained from a natural source or manufactured synthetically. Alternatively, the antigen may by produced by a recombinant DNA vector in *E. coli* or another bacterial or nonbacterial host. Samples for the assays may be body fluids or other tissue samples from humans or animals. The presence of reactive antibodies in the samples may be demonstrated by antibody binding to the diagnostic component followed by detection of the antibody-antigen complex by any of a number of methods, including ELISA, RIA, fluorescence, agglutination or precipitation reactions, nephelometry, or any of these assays using avidin-biotin reactions. The degree of reactivity may be assessed by comparison to control samples, and the degree of reactivity used as a measure of present or past infection or disease. The assay(s) could also be used to monitor reactivity during the course of a disease, e.g., to determine the efficacy of therapy.

The term "a substantial immunological reactivity" is meant to designate a marked immunological binding between an antibody/antiserum on the one hand, and on the other a diagnostic component under well-defined conditions with respect to physicochemical parameters as well as concentrations of diagnostic component. Thus, a substantial immunological reactivity should be clearly distinguishable from a non-specific interaction between an antibody/antiserum and a diagnostic component. This distinction can, for instance, be made by reacting the antibody/antiserum with a known concentration of a diagnostic component which has previously been shown not to react with the antibody/antiserum, and then using this reaction as a negative control. A positive control

could suitably be the reaction between the antibody/antiserum and the same concentration of the diagnostic component used for the immunisation resulting in the production of the antibody/antiserum.

By the term "epitope" is meant the spatial part of an antigen responsible for the specific binding to the antigen-binding part of an antibody or of a T-lymphocyte.

By the term "polypeptide" is understood a molecule comprising at least two amino acids joined by a peptide bond. The term polypeptide thus indicates small peptides (less than 10 amino acid residues), oligopeptides (between 10 and 100 amino acid residues), proteins (the functional entity including at least one peptide and/or prosthetic groups and/or glycosylation and/or lipidation, such as lipopolypeptides and glycopolypeptides, etc.) as well as traditional polypeptides (more than 100 amino acid residues). Interesting polypeptides for linkage to the branched polymer of the invention are recombinant polypeptides which may be prepared in accordance with methods generally known.

Particularly interesting molecules which may be used alone or in combination with the linking of other molecules to the peptide of the invention and the linking of which therefore constitute an important aspect of the invention are mediators or immunomodulators such as cytokines or bioactive cytokine sequences which have important functions in the regulation of the immune response, in particular T-cell reactions.

A particularly interesting aspect is the use of recombinant, synthetically prepared, or native mediators, e.g cytokines or a part thereof having cytokine activity inserted into immunogenic complexes such as Iscoms or other carriers such as microparticles together with a peptide of the invention to which at least one other molecule is linked. Other combinations may be immunogenic complexes carrying cytokines or a part thereof having cytokine activity in mixtures with

immunogenic complexes carrying branched peptide-construct or it may be immunogenic complexes carrying peptides of the invention to which an active part of an immunomodulator is linked in combination with other peptides. Among the cytokines which may be of relevance are interleukin 1 - 18, TNF (tumour necrosis factor), lymphotoxin, and interferon-alpha, -beta, -gamma. Alternatively, bioactive cytokine-specific sequences having cytokine activity may be part of the sequence linked to the peptide of the invention.

A vaccine composition prepared using a vaccine component in which an immunogenic agent is attached to the peptide of the solid phase complex of the invention is also part of the invention, the amount of the vaccine component being effective to confer substantially increased resistance to the infection in question in an animal, including a human being, against an infectious agent such as a bacteria, a virus or a parasite as compared the degree of resistance present in an animal not previously exposed to the infectious organism. Thus, the invention also relates to the use of an immunogenic component for preparing a vaccine composition and to a method for immunizing an animal, including a human being, against an infectious organism using the vaccine composition of the invention.

Suitable immunogenic agents include polypeptides, carbohydrates, lipids or nucleotide sequences alone or in various combinations, optionally together with other substances, such as an immunomodulator mentioned above or a chemical compound capable of increasing the immunogenic effect to the vaccine component. Interesting aspects of the invention include various combinations of immunogenic agents derived from different infectious organisms whereby a vaccine composition capable of conferring increased resistance to several infectious organisms is obtained.

The vaccine composition may optionally be formulated in combination with a pharmaceutically acceptable carrier or

vehicle and the vaccine optionally further comprising an adjuvant.

By the term "conferring substantially increased resistance to infections" is meant that the administration of the vaccine composition to the animal has the effect that disease caused by infections with an infectious agent is avoided or diminished or at least that the risk of catching the disease is significantly reduced.

Typically, the vaccine composition according to the invention are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The vaccine composition is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine composition.

The vaccine compositions are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules,

sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The vaccine composition may be formulated as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccine compositions are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner. However, suitable dosage for humans are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 1 μg to 500 μg, especially in the range from about 10 μg to 50 μg. Suitable regimes for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like, including nasal application and application on other suitable body surfaces. The dosage of the vaccine will

depend on the route of administration and will vary according to the size of the host.

Various methods of achieving adjuvant effect for the vaccine composition of the invention include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gramnegative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably two or three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain levels of the antibodies. The course of the immunization may be followed by assays for antibodies for the antigens. The assays may be performed by labelling with conventional labels, such as radionuclides, enzymes, fluorescers, and the like. These techniques are well known in the art.

It is contemplated that the vaccines of the invention should be effective in activating both arms of the immune system. Thus, vaccines capable of eliciting a cell-mediated immune reaction are also a part of the invention.

Methods of actively immunizing animals, including mammals such as human beings against at least one infectious agent with a vaccine according to the invention are also parts of the invention. The methods generally consist of the administration to the animal of an immunogenically effective amount of the vaccines of the invention.

The invention further relates to a therapeutic component comprising the peptide of the solid phase complex of the invention to which at least one therapeutic or prophylatic agent capable of treating or preventing a disease, such as an infectious, cancerous or autoimmune disease is linked. The therapeutic agent may be any agent capable of acting as a prophylactic agent, a therapeutic agent, or as an agent in the prevention or relapse of a disease or in the prevention or disruption of pregnancy. The agent may be a polypeptide, a glycopeptide, a lipopeptide, a phospolipid, a polysaccharide, a lipopolysaccaride, a carbohydrate, a nucleotide sequence or any combination or modifications thereof.

In a particular interesting embodiment of the invention, a therapeutic component comprises one or more therapeutic or prophylactic agents in combination with at least one immunogenic substance or immune mediator capable of controlling or enhancing the effect of the therapeutic or prophylactic agent. The immunogenic substance or immune mediator may be any molecule such as a carbohydrate or a peptide or a nucleotide which can be naturally, synthetically or recombinantly derived, or the mediator may be a chemical compound.

The invention also relates to any therapeutic composition containing a therapeutic component of the invention. Therapeutic compositions comprising one or more therapeutic components to which different therapeutic agents capable of preventing or treating the same or different diseases or disrupting pregnancy constitute another aspect of the invention. The invention further relates to the use of a therapeutic composition of the invention in the prevention or treatment

of a disease, or the prevention or disruption of pregnancy. The therapeutic composition may be administered in any convenient way, including intramuscularly, subcutaneously, intradermally, orally, nasally, and intravenously.

One interesting aspect of this part of the invention consists of a therapeutic composition comprising a therapeutic component of the invention attached to a carrier, such as an immunostimulating complex in combination with at least one mediator or a part thereof having mediator activity. The mediator or a part thereof having mediator activity may be linked to the peptide of the invention and may be further attached to an immunostimulating complex which may be the same or different from the immunostimulating complex carrying the therapeutic component. Particular interesting mediators include immunomodulators, such as cytokines including interleukins, interferons, tumour necrosis factors or a part thereof having immunomodulator activity.

Another interesting aspect of this part of the invention comprises a therapeutic component to which a molecule capable of binding to a target molecule present at a specific location in a body, including a human being, is attached thereby directing the therapeutic component to said specific location at which the therapeutic component is to exert its effect. The molecule capable of binding to a target molecule provide means for targeting the activity of the therapeutic agent. Antibodies directed against and capable of binding to the target molecule constitute interesting examples of such molecules.

Another aspect of the invention relates to a pharmaceutical composition for the prevention after establishment of being in a high risk group of developing an autoimmune disease, treatment or prevention of a relapse of an autoimmune disease, a cancerous disease or an infectious disease caused by an infectious agent, said composition comprising the branched polymer of the invention to which a therapeutic agent or a

combination of various therapeutic agents are linked. The pharmaceutical composition may be formulated according to known methods based on pharmaceutically acceptable excipients.

Furthermore, the invention relates to the use of a pharmaceutical composition according to the invention for combatting, prevention or treatment of infectious agents, autoimmune disease or cancerous diseases.

The non-dendritic peptides may be used without addition of adjuvant or in combination with adjuvants like Freund's adjuvant and aluminium hydroxid or with and without insertion into immunostimulating complexes (Iscoms) or liposomes.

For immunization purposes, any mouse strain (inbreed and outbreed), rabbits, guinea pigs, minks or other animals may be used. Both humans and animals may be vaccinated with the non-dendritic peptide. The number of and intervals between immunizations may be varied. The non-dendritic peptide carrier-immunogenic complex according to the present invention may be used for ip., sc., im., iv., oral, nasal, anal, vaginal etc. immunizations. Different amounts of immunogenic peptides may be used. In addition, the non-dendritic peptide carrier-immunogenic complex may also be used for the induction of amamnestic responses.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention relates to a non-dendritic peptide carrier coupled e.g. by its C-terminus through a specifically cleavable linker to a solid phase forming a peptide carrier-solid phase complex. The non-dendritic peptide carrier preferably comprises about 10-50 amino acids capable of forming a secondary structure in a benign buffer after liberation from the solid phase, the peptide complex further comprising an immunogenic substance coupled thereon. The secondary structure in the benign buffer

results in a stable carrier function of the non-dendritic peptide when liberated from the solid phase. The immunogenic substance coupled to the non-dendritic peptide include antigenic substances which is covalently coupled thereon.

The secondary structure may be any structure selected from the group α-helices, β-strand, β-turns, γ-turns, zinc-finger structures as well as combinations thereof. The primary function of the structure is to secure that the immunogenic substance is presented to the environment in a stable and predictable form. Accordingly, any of the secondary structures which in the given circumstances provides a suitable orientation of the immunogenic substance is within the scope of the invention.

The amphipathic α-helix in general is a well-studied (e.g. Mant 1993) and relatively simple peptide structure that retains a well-defined conformation in water. An amphipathic helix has a hydrophobic side occupying one half of the helix along its axis and a hydrophilic side on the other half. Amphipatic α-helices typically combine in "bundles" shielding the hydrophobic faces and exposing the hydrophilic side to form parallel or antiparallel homodimers (Zhu 1993) or heterodimers and oligomers (Zhu 1992).

Amphipathic helices are generally characterized by "heptads" dictated by the periodicity of the α-helical turn, in which positions a, b, c, d, e, f, and g are occupied by amino acid residues following some simple rules: Positions a and d are mainly occupied by hydrophobic amino acids (especially leucine, isoleucine, and valine, and to a lesser extent alanine, phenylalanine, tyrosine, methionine, and tryptophan) while other positions are free to any other residue except proline and glycine; there may be a bias towards residues with high helix propensities (alanine, arginine, leucine, lysine, methionine, glutamine, glutamic acid, isoleucine).

The exact arrangement by which a pair of α-helices combines to form an inter-molecular complex can be further restricted by additional design rules (see e.g. Mant 1993 and Zhu 1993), e.g. by selecting a/d residues of varying hydrophobicity and size (alternating "small" and "big" (e.g. alanine and leucine respectively) destabilises parallel packing and stabilises anti-parallel packing) and by either choosing e and g positions of opposite charges (favouring a parallel orientation) or of the same charge (favouring an anti-parallel orientation) (see e.g. WO95/31480 and Chang 1994).

It is evident that this kind of structure is particularly robust and versatile with a attractive degeneracy increasing the freedom of design (see e.g. the "semirandom" design by Kamtekar (1993). The peptide lenght required to form a helix is in the practical range of chemical synthesis (minimal lenght is around 15-20 amino acids (Kamtekar 1993, Bryson 1995, Fezoui 1995)). Metal ions chelated by H-XXX-H histidine pairs in an α-helix (see e.g. Regan 1995) can be used to probe and stabilize α-helical structures.

Secondary structures can further be stabilised by metal-ion-chelation as well as by lactam- or disulfide bridges.

Non-dendritic peptide carriers according to the invention may also include mixtures of two or more different secondary structures. Examples of such mixed structures will often include α-helices, e.g two α-helices joined by a β-turn. In addition, the non-dendritic peptide may in part include a peptide with no well defined secondary structure selected from the group of random coil, Ω-loop and undefined loop or combinations thereof. The peptide or peptide fragment having no well defined structure has no influence on the non-dendritic peptide carrier's capability of forming a secondary structure in a benign buffer. The number of amino acids in the part having no secondary structure contributes to the overall number om amino acids of the non-dendritic peptide carrier according to the present invention.

A specific and well-studied metal-ion stabilized, composite structure is "zinc-finger" domains characteristic of DNA-binding proteins, having the general structure Y,F-X-C-$X_{2,4}$-C-X_3-F-X_3-H-$X_{3,4}$-H, where X can be any amino acid (Krizek 1991) and where Zn^{++} is tetrahedrally coordinated by the C and H residues of two different strands. The resulting Zn-supported ßßα-structure is stable in water. A 26 amino acid residues consensus-sequence has been defined by Krizek (1991-)(PYKCPECGKSFSQKSDLVKHQRTHTG) and used by Bianchi (1995) for creating combinatorial libraries exploiting the stability of the α-helix to substitutions along the peptide chain. Struthers (1996) designed a 23 amino acid-residue conformationally stable structure based on the Zn-finger sequence but not needing Zn-support.

In one embodiment, the peptide carrier is in the form of a non-dendritic peptide which forms an amphipathic helix in a benign aqueous solution as the result of an intramolecular anti-parallel arrangement of two α-helices joined by a turn in a monomer super-secondary hairpin structure.

It is preferred that the non-dendritic peptide is stabilised by the chelation of divalent metal-ions selected from Cu++, Co++, Zn++, Ca++, Ni++, and Cd++.

In general, the secondary structure of the non-dendritic peptide carrier is obtained by the result of the inclusion in the peptide of one or more α-helical -, β-strand-, turn- or zinc-finger inducing sequences of amino acids, respectively, or combinations thereof.

In one embodiment, the secondary structure may be induced by structure-nucleating molecular building blocks selected from

i) the α-helix inducers aminoisobutyric acid, acetylpropyl-prolin with a thiamethylen bridge and HX_3H, any of the H-residues being replaceable by C, chelating a divalent metal ion; and

ii) the β-strand inducers 4-(2-aminoethyl)6-dibenzofuran and diacylaminoepindolidione and HXH, any of the H residues being replaceable by C, chelating a divalent metal ion; and
iii) the β-turn-inducers (S)-α-methylproline and HX_2H, any of the H residues being replaceable by C, chelating a divalent metal ion; and

iv) generally stabilizing molecular building blocks as proline and thiazolidine.

In a further embodiment, the peptide is linked by its C-terminus to a dibranching molecule presenting two molecules of the peptide in parallel. The dibranching molecule may be known in the art such as ornithine and lysine.

In a preferred embodiment, the non-dendritic peptide carrier according to the invention gives rise to parallel homodimers of the α-helix coiled-coil type.

For the attachment of the immunogenic substance, the non-dendritic peptide carrier according to the invention in one embodiment carries at least 2 attachment points, generally defined as derivatizable and accessible functional groups. These functional groups include hydroxylamine-, amino-, hydrozy-, thiol-, haloacetyl-, carbonyl-, α-oxoacyl, 1,2-thiolamin-, acylhydrazine, alkylthiol-, acylthiol, carboxylate-groups, and mixtures thereof. The number of attachment points is selected in accordance with the desired number of immunogenic substances of the final product, accordingly, the non-dendritic peptide may comprise at least 4 attachment points or any other desired number which is possible according to the lenght of the peptide.

The attachment points may be functional groups well known in the art. Preferred attachment points are chosen from the group of ε-amino groups or derivatized ε-amino groups in the side-chain of lysine and a free α-amino group. In one aspect, the non-dendritic peptide carrier according to the invention

comprises as attachment points, ε- and/or α-amino groups of a lysine residue coupled to the ε-amino group of the lysine residue of the original attachment point, thereby leading to a double-functional attachment point, or, by further derivatization with lysine residues to a multiple-functional attachment point. One of the functional groups of the double-functional attachment points, or at least one of the functional groups of the multiple-functional attachment points, may be blocked in one aspect. The blocking may be by a protecting group cleavable by a chemical treatment such as orthogonal to both Fmoc- and Boc-cleaving chemical treatments. The protecting groups include Dde (1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-ethyl-) and allylic protecting groups such as Aloc (allyloxycarbonyl)

The advantage of the procedure described above is the introduction of a so-called "orthogonal" strategy for α-amino protection and side-chain functionality-protection and solid-phase linkage, respectively. "Orthogonal chemistries" means that one type of protecting group is stable to the chemical treatment used for the cleavage of the other and vice versa. One such very popular strategy employs base-labile fluorenylmethoxycarbonyl (Fmoc) as an α-amino protecting group and acid-labile protecting groups for side-chains and acid-labile "linkers". A range of linkers with graded labilities towards, e.g., acids, bases, water, etc., are known, leading to the liberation of the peptide either as a free acid or as a specific derivative, most typically an amide.

The non-dendritic peptide carrier according to the invention may also comprise two or more amino groups located in a side-chain attached to the C-terminal amino acid.

In a further embodiment, a non-dendritic peptide carrier according to the invention may comprise a subclass of ε-amino groups in the side-chain of other lysine residues and a free α-amino group, said subclass comprising at least one such amino group being protected by a protecting group cleavable

by a chemical treatment orthogonal to both Fmoc- and Boc-cleaving chemical treatments.

In one aspect, the amino groups serving as attachment points may further be derivatized by chemical means selected from

i) treatment with di-acid anhydrides, preferably succinic anhydride to yield free side-chain carboxylic acids, and

ii) treatment with haloacetic acid anhydride, preferably bromoacetic acid anhydride to yield free haloacetyl groups, preferably bromoacetyl groups, and

iii) treatment with activated maleimide, preferably m-maleimide benzoyl-N-hydroxysuccinimide ester to yield free maleimide groups, or treatment with SPDD to yield free pyridyldithio grops, and

iv) treatment with carboxy-activated α-amino-protected cysteine to yield free thiol groups, and

v) treatment with carboxy-activated α-amino-protected cysteine to yield free thiol groups, followed by deprotection of the α-amino group to yield free 1,2 thiol-amino groups, and

vi) treatment with glutamic or aspartic acid to yield free side-chain carboxylic groups, and

vii) treatment with amino-oxyacetic acid to yield free hydroxylamine groups, and

viii) treatment with α-amino-protected, carboxy-activated serine to yield, after α-amino deprotection free 1,2-aminoalcohols, which in turn can be oxidised to free α-oxoacyl groups, and

ix) treatment with Boc-monohydrazide succinic acid to yield free acyl-substituted hydrazines.

The non-dendritic peptide carrier according to the invention may also comprise side-chain functional groups serving as attachment points which are derivatized with secondary structure supporting, derivatizable building blocks, selected from

i) the α-helix inducers aminoisobutyric acid, acetylpropylprolin with a thiamethylen bridge and HX_3H, any of the H-residues being replaceable by C, chelating a divalent metal ion; and

ii) the β-turn-inducers (S)-α-methylproline and HX_2H, any of the H residues being replacable by C, chelating a divalent metal ion; and

iii) the β-strand inducers 4-(2-aminoethyl)6-dibenzofuran and diacylaminoepindolidione and HXH, any of the H residues being replacable by C, chelating a divalent metal ion; and
iv) generally stabilizing molecular building blocks as proline and thiazolidine.

The side-chain functional groups of the non-dendritic peptide carrier serving as attachment points may, in one embodiment, be derivatised with a dibranching residue being selected from the structures disclosed in Fig. 4A and Fig. 4B.

The non-dendritic peptide may, in one aspect, comprise at least two free carboxylic acids or amino groups located in a side-chain attached to the C-terminal amino acid, preferably a lysine, or said side-chain attached near the C-terminal amino acid, the said non-dendritic peptide further comprising other attachment points not constituted by carboxylic acids. One example of a method for preparing a non-dendritic peptide comprising at least two carboxylic groups in a side chain attached to or near the C-terminal amino acid comprises introducing one or multiple glutamic acid residues or

aspartic acid residues preferably by synthesis on the ϵ-amino group of one selected C-terminally located lysine residue using orthogonal protection for the α-amino group, compared to the protection use for the amino acids incorporated in the rest of the peptide and demasking the side-chain carboxylic groups before proceeding with synthesis of the backbone peptide.

Similarly, a method for preparing a non-dendritic peptide comprising at least two amino groups in a side-chain attached to or near the C-terminal amino acid comprises introducing one or multiple lysine residues preferably by synthesis on the ϵ-amino group of one selected C-terminally located lysine residue using orthogonal protection for the α-amino group, compared to the protection use for the amino acids incorporated in the rest of the peptide and not demasking the side-chain carboxylic groups before proceeding with synthesis of the backbone peptide.

To further ameliorate the immunogenicity and versatility of the immunogenic response, various lipids with adjuvant effects may be included in the immunogenic compounds according to the invention. The basic design according to the invention has been developed to adapt a number of different antigens and other entities such as alkyl chains in the structure.

Accordingly, in a further aspect, the non-dendritic peptide carrier may comprise one or more alkyl-chains, preferably in the form of a saturated fatty acid bound covalently to the N-terminus of the peptide or to an amino acid side-chain. The carbon chain of the alkyl-chain mentioned above may comprise about 4-25 carbon atoms such as 6-20 carbon atoms, preferably 7-17 carbon atoms in length. In a preferred aspect, the carbon chain is a lipidic moiety comprising palmitic acid or myristic acid or mixtures thereof. An example of such lipid moiety is the immunostimulatory $palm_3$-Cys-molecule as shown in Fig. 4C.

In one interesting embodiment, a non-dendritic peptide carrier according to the invention comprises at least one lipidic moiety bound as a thioester to the peptide, preferably through a cysteine side chain thiol group.

In an aspect the lipidic moiety is provided by fatty acids, typically palmitic acid or myristic acid coupled to the side chains of amino acids selected from lysine and serine. The lysine and serine may constitute a linear peptidic chain and preferably being of alternating chirality, a D-amino acid being followed by a L-amino acid and a L-amino acid being followed by a D-amino acid.

In a further embodiment, the non-dendritic peptide carrier comprises a lipidic moiety such as palmitic or myristic acid located at the C-terminus or near the C-terminus. In one aspect, it is preferable that the peptide comprises no free and accessible side-chain functionalities but an unprotected N-terminus α-amino group.

An aspect of a non-dendritic peptide carrier according to the invention is a carrier comprising 20-50 amino acids and which comprises in its sequence repeated "heptads" having the form "abcdefg". Preferably, the positions "a" and "d" are occupied by hydrophobic amino acids selected from I, V, L, F, and A, positions "e" and "g" by charged amino acids of opposite signs, selected from E, D, and K. Positions "b", "c", and "f" are preferably occupied by any amino acid residue selected from the group of A, R, N, D, E, C, Q, E, H, I, L, K, M, F, S, T, W, Y or V but preferably A, S, T, C, H or K, yielding 2-10, preferably 4-6 evenly spaced lysine residues with freely accessible derivatizable ϵ-amino groups. In a preferred embodiment, at least one pair of histidines or a histidine and a cysteine are occupying consecutive "b" and "f" positions in the peptide chain.

In one embodiment, the repeated "heptads" are located in its inner sequence, i.e. the sequence located within the segment

of the peptide corresponding to the segment from amino acid 2-5 to n-(2-5), preferably to the segment from amino acid 3 to n-3, where n is the number of amino acids in the peptide. In a further aspect the lysine residues occupying "e" and "g" positions are orthogonally side-chain protected compared to lysine residues occupying "b", "c", and "f" positions. The orthogonal protecting group is preferably being stable towards treatments, such as Fmoc- as well as Boc-cleaving treatments. In another aspect, at least one "f" position is occupied by C.

The carrier may also comprise at least one, preferably one lactam bridge linking a "b" and an "f" amino acid in a terminally placed heptad, "b" and "f" being either E and K or K and E, E being in both cases replaceable by D. In addition, the carrier may comprise one Y or W included in the sequence, preferably as one of the 2-5 C-terminal amino acids.

T-cell help is important for efficient immunizations. T-cells are activated by interaction with peptides bound by the major histocompatibility complex (MHC) on antigen-presenting cells. The MHC of an individual bind a subset of peptides conforming to the sequence definition of that individual only (see Rammensee, 1995). "Promiscous" peptides have been described that are bound by a large number of individual MHCs covering a large proportion of the species in question. The art of incorporating T-cell stimulating moieties in synthetic peptide constructs intended for immunizations is well known. In addition to general "stimulators" including tuftsin (-=[Thr-Lys-Pro-Arg]$_N$)(e.g. Fridkin 1989), muramyldipeptide (MDP = N-acetyl muramyl L-alanyl D-isoglutamine)(Ellouz 1974), and lipopeptides (Wiesmüller 1992) or simple lipids (Flinn 1994, Vitiello 1995) a number of "promiscous" peptides, binding to a large subset of MHC-molecules have been described (Panina-Bordignon 1989). These substances can be combined with peptide antigens in linear (e.g. WO 95/00540, Wang 1995, Vitiello 1995 and Kaumaya 1993) or branched arrangements (US patent 5229490, WO 93/22343, Pawan 1994, Flinn

1994, Jackson 1995). The lipid-structure by Wiesmüller (1992) is particularly efficient (Defoort 1992) and has even been shown to be efficient in a linear construct with a dimeric carbohydrate antigen (Toyokuni 1994). Importantly, a "mixed" linear poly-epitope (recombinantly expressed), containing only core sequences of several T-cell stimulatory peptides has been found to stimulate all subsets binding the individual peptides (Thomson 1995). It was not shown but may be possible to inject such DNA-constructs directly by the known art of DNA-immunization (e.g. Ulmer 1993) and obtain a similar broad stimulation in this way.

Peptide-attachable DNA-binding substances include intercalators (e.g. quinoline (Brown 1994)) which bind DNA unspecifically and peptide nucleic acids (PNA) which can hybridise with DNA in a sequence-specific way (WO 95/01369).

Also, specific imunomodulators, e.g. cytokines or cytokine fragments, may be included (e.g. Kumaratilake 1995, European Patent 0 604 727 A1).

Accordingly, to circumvent restriction of the Major Histocompability Complex, specific T-cell stimulatory peptides, in addition to the immunogenic substance, may be included in the immunogen complex according to the invention. This may be done by peptide synthesis using orthogonal chemistry or by attaching composite linear constructs.

Accordingly, a non-dendritic peptide according to the invention may comprise one or more copies of a peptide moiety or comprising a combination of peptide moieties. The peptide moiety may in one embodiment be situated at either or both ends of the non-dendritic carrier in its side-chain- and N-terminal-blocked form. Examples of such peptide moieties include the following:

i) $[TKPR]_N$, in which N is preferably from 1-5 (tuftsin oligomer), muramyldipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine) or variants thereof, and

ii) a T-cell stimulatory peptide selected from QYIKANSKFIGITE (tetanus toxoid 830-843) and FNNFTVSFWLHRVKVSASHLE (tetanus toxoid 947-967), DQVHFQPLPPAVVKLSDALI (Mycobacterium tuberculosis 38 kD antigen 350-369), DIEKKIAKMEKASSVFNVVNS (Plasmodium falciparum circumsporozoite protein 378-398), KLLSLIKGVIVHRLEGVE, measles virus F-protein 286-302, LDNIKGNVGKMEDYIKKNNK (Plasmodium falciparum MSP-1, 260-279), LQTMVKLFNRIK, NSVDDALINSTKIYSYFPSV, QYIKANSKFIGITELK, and PGINGKAIHLVNNESS; and

iii) a T-cell stimulatory peptide selected from poly T-cell-epitope constructs wherein the T-cell-epitope elements are arranged in a substantially linear construction comprising interposed minimal T-cell epitope peptide segments, preferably without flanking sequences; and

iv) a cytokine derived peptide selected from
IFN-gamma(1-39) HGTVIESLESNNYFNSSGIDVEEKSLFLDIWRNWQKDG,
IFN-gamma(95-133) AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC,
TNF (70-80) PSTHVLITHTI
IL-1 beta (163-171) VQGEESNDK;
and combinations thereof.

In one embodiment, the non-dendritic peptide carrier may comprise the specified peptide moiety in its side-chain protected, but N-terminally unprotected form, said peptide moiety being coupled as a branch peptide to at least one side chain attachment point of a backbone peptide. An example of such attachment point is an ϵ-amino group in the side chain of lysine.

In a further embodiment, the non-dendritic peptide comprises the specified peptide moiety in its side-chain protected, but N-terminally unprotected form, said peptide moiety being

coupled as a branch peptide to at least one side-chain attachment point in a backbone peptide, said attachment point being a double-functional or multiple-functional attachment point. Relevant groups may be ϵ- and α-amino groups of a lysine residue coupled to the ϵ-amino group of the lysine residue of the original attachment point,

In yet another embodiment, the non-dendritic carrier comprises the specified peptide moiety in its side-chain protected and N-terminally protected or N-terminally unprotected form, said peptide moiety being coupled to at least one side-chain attachment point in a backbone peptide. The attachment point may be a double-functional or multiple-functional attachment point and in a further embodiment wherein at least one are further protected orthogonally to Fmoc and Boc and other acid-labile side-chain protecting groups.

The non-dendritic peptide according to the invention may also comprise at least one PNA moiety. The PNA may comprise a monomer sequence binding a specific DNA-molecule by hybridization. In another embodiment, the non-dendritic peptide comprises at least one functional DNA-intercalator moiety. This intercalator is in one embodiment quinoline.

In addition, the non-dendritic peptide carrier may comprise at least one DNA or RNA oligonucleotide moiety. The DNA oligonucleotide moiety is preferably one which is coupled through its 3'-end to an amino group of said peptide. Furthermore, it may comprise a nucleotide sequence encoding a T-cell stimulatory peptide. In a preferred embodiment, the oligonucleotide sequence binds a specific DNA- or RNA-molecule by hybridization.

In a preferred embodiment, the attached oligonucleotide comprises a hexanucleotide corresponding to the general formula (5')PuPuCGPyPy(3') in which Pu is a purine base, Py is a pyrimidine base and CG represents unmethylated CpG dinucleotide. This hexanucleotide motif has been shown to be

a potent inducer of cytokines in a range of immunocompetent cells (Klinman 1996).

The non-dendritic peptide carrier may comprise a spacer molecule. Preferably, the spacer is selected from G_N, where N=2-8.

The solid-phase on which the non-dendritic carrier is coupled is preferably a polymer whereby the solid phase complex represents a copolymer. It may be advantageously to bind the non-dendritic peptide to the solid phase through a linker which is selectively cleavable by a specific chemical treatment.

The solid phase is normally constituted by derivatizable polymers (matrices, resins), which are insoluble in water and in organic solvents including dimethylformamide, dimethylsulfoxide, N-methylpyrrolidone, dichloromethan, piperidine, diethylether and aqueous trifluoroacetic acid. Divinylbenzene/polystyrene and polyacrylamide polymers sometimes in combination with macroscopic support materials such as kieselguhr and derivatized with a variety of spacers and linkers. The amount of peptide on the polymer may vary according to size of the peptide or the specific conditions of the synthesis.

In one aspect, the non-dendritic peptide carrier may be substituting the solid-phase polymer to about 0.001 to about 5, preferably such as from about 0.01 to about 1, more preferred from about 0.02 to about 0.08, still more preferred from about 0.04 to about 0.06, most preferred from about 0.05 to about 0.1 mmoles pr. gram of solid phase.

The chemical treatment liberating the non-dendritic carrier peptide from the solid-phase bound linker may be orthogonal to the chemical treatment used for the cleavage of protecting groups from the intended attachment points in the side-chains of the peptide. Such linkers may be selected from 3-nitro-4-

hydroxymethyl benzoic acid type linkers cleavable by photolysis, hydroxy-crotonyl-aminomethyl type linkers cleavable by catalytic hydrogenation, 4-methylbenzhydrylamine type linkers cleavable by hydrofluoric acid or trifluoromethanesulfonic acid, unmodified Rink-type linker (4-(2', 4'-dimethoxyphenyl Fmoc-aminomethyl)-phenoxyacetic acid) cleavable by aqueous trifluoroacetic acid.

In one embodiment, the non-dendritic carrier peptide is bound as an ester to a linker to the solid-phase said peptide being cleavable by aqueous base.

The non-dendritic carrier peptide may also be linked to a solid-phase bound 4-hydroxymethylbenzoic acid liberating the peptide as a carboxylic acid upon treatment with hydroxyl-nucleophiles, as an amide upon treatment with methanolic ammonia and as a hydrazide upon treatment with hydrazine.

The non-dendritic peptide carrier according to the invention may, in one embodiment, be linked to the solid phase through a linker releasing the peptide on contact with water, preferably a glycolic acid linker of the structure given in Fig. 4D or a linker releasing the peptide through diketopiperazine formation.

In an aspect of the invention, the non-dendritic peptide carrier in a solid-phase complex comprises 5-50, preferably 10-20 amino acids, the peptide also comprising a covalently bound lipidic moiety, preferably palmitic or myristic acid which is located at the N-terminus or near the N-terminus, the peptide being attached to the solid phase by a hydrazine-clevable linker to form the solid phase complex.

In order to detect the non-dendritic peptide carrier according to the invention, it may comprise a substance having characteristic and measurable spectral or radioactive properties such as UV-absorbing properties, visibly absorbing or fluorescent properties.

In a further embodiment, the invention is directed to the use of a non-dendritic peptide carrier as defined above as a scaffold for carrying other moieties by their covalent attachment to derivatizable groups of the said peptide. The derivatizable group is in one embodiment selected from an α-amino group, an ε-amino group of K, a chemically prepared derivative of an ε-amino group of K, and a thiol group from the side-chain of C.

In yet another embodiment, the invention is directed to the use of a non-dendritic peptide carrier as defined above as a scaffold for the production of chemical derivatives, characterised by covalently attached molecules at the attachment points. The molecules may be selected from peptides, carbohydrates, haptens, glycopeptides, lipopeptides, DNA, RNA, PNA, proteins and glycoproteins and combinations thereof.

In an additional embodiment, the invention is directed to the use of a non-dendritic peptide carrier as defined above coupled to a solid phase as a solid-phase complex scaffold for the stepwise conventional Fmoc- or Boc-based solid-phase peptide synthesis for the stepwise synthesis of peptide moieties with defined sequences on the attachment-points of the solid-phase bound peptide followed by specific cleavage of the whole complex from the solid phase.

Components which can be coupled on the non-dendritic peptide carrier according to the uses defined above may be selected from chemically synthesized protected peptides, chemically synthesized unprotected peptides, recombinant synthesized peptides, naturally-derived peptides, naturally-derived or synthetic carbohydrates, naturally-derived or synthetic glycopeptides, naturally-derived or synthetic lipopeptides, naturally-derived or synthetic nucleic acids, naturally-derived or synthetic ribonucleic acids, peptide nucleic acids, haptens or other antigens or non-antigens, or other amino-, carboxylate-, haloacetyl-, maleimide-, thiol-, 1,2-

thiol-amino-, hydroxylamine-, α-oxoacyl-, carbonyl-, and acylhydrazine-reactive substances and mixtures thereof.

The non-dendritic peptide carriers of this invention can also be used for the production of structured synthetic peptide libraries which in contrast to normal peptide libraries is characterized by a higher degree of conformational definition achieved by a structure-supporting framework in the present invention provided by the non-dendritic peptide carrier. Furthermore, the libraries that can be achieved by the present invention can be used for immunization. Thus, one particular aspect of peptide libraries derived from non-dendritic peptide carriers according to the present invention is that a structured library is obtained which is recognized easily by natural antibodies. In addition, the library can be used for immunization with the aim of achieving broad protection e.g. against a range of pathogenic virus variants.

The non-dendritic peptide carrier can be used in standard methods of preparing peptide libraries, including the split-combine approach which results in a population of derivatized non-dendritic peptide carrier molecules in which only identical branch peptides are coupled to the same non-dendritic peptide carrier molecule.

The non-dendritic peptide carrier according to the invention may be used as a scaffold for the coupling of one or more components selected from chemically synthesized protected peptides, chemically synthesized unprotected peptides, recombinant synthesized peptides, naturally-derived peptides, cyclic peptides, naturally-derived or synthetic carbohydrates, naturally-derived or synthetic glycopeptides, naturally-derived or synthetic lipopeptides, naturally-derived or synthetic nucleic acids, naturally-derived or synthetic ribonucleic acids, peptide nucleic acids, haptens or other antigens or non-antigens, or other amino-, carboxylate-, haloacetyl-, maleimide-, thiol-, 1,2-thiol-amino-, hydroxylamine-, α-oxoacyl-, carbonyl-, and acylhydrazine-

reactive substances and mixtures thereof. This use as a scaffold can be performed when the non-dendritic peptide carrier is liberated from the solid phase. However, it is preferred that the coupling of one or more components as mentioned above is performed with the non-dendritic peptide carrier still attached to the solid phase.

The peptide carrier-solid phase complex according to the invention may also be used as a scaffold for the attachment of moieties through the C-terminally located carboxylic acids in the said peptide carrier-solid phase complex, said attached moieties being typically amine-containg compounds, most typically synthetic or natural peptides or proteins with which a selective coupling through their amino groups can be achieved by known methods for conjugating peptides and proteins to proteins, then cleaving off protecting groups of the backbone lipopeptide as well as of the coupled peptides if any, followed by liberation of the whole complex.

A further method of using the non-dendritic peptide carrier-solid phase complex according to the invention is by combinating it with a free peptide acid preferably comprising 5-20 amino acids and containing a lipidic moiety located at the N-terminus or near the N-terminus, said lipidic moiety being preferably palmitic or myristic acid, said peptide further carrying no other free functional groups than the C-terminus carboxylic acid. The method comprises coupling, preferably by sequential peptide synthesis a target peptide to the peptide carrier-solid phase complex, terminating the synthesis by coupling the free lipopeptide to the α-amino group of the solid-phase bound peptide and subsequently cleaving off protecting groups and liberating the whole complex, which then, upon liberation will expose the synthesized target peptide as a loop. The peptide structure may be stabilized by including a moiety bridging the two ends of the peptide by a structure selected from an intramolecular lactam-linkage, an intramolecular S-S-linkage and a suitable bifunctional spacer molecule.

The lipopeptide hydrazide deriving from hydrazine-cleavage of peptide carrier-solid phase complex may be used for coupling aldehyde-containing compounds, such as reducing carbohydrates which may be naturally-occuring or synthetic carbohydrates or such carbohydrates mildly oxidized to accomplish ring opening, said method consisting of mixing said reducing carbohydrate with the lipopeptide hydrazide.

Glycoconjugates chosen from glycoproteins, glycopeptides or glycolipids, which may be glycosylated immunomodulatory glycoconjugates selected from interleukin 6, interferon-γ, other glycosylated cytokines and immunoglobulins may be coupled selectively through its carbohydrate moiety to the hydrazide group of the free lipopeptide by the above method.

A non-dendritic peptide carrier immunogen complex may be incorporated into Immunostimulating Complexes (Iscoms) or liposomes by virtue of the lipidic moiety resulting in a non-dendritic peptide carrier-Iscom complex.

Two or more different complexes may be incorporated into the same Iscom or liposome. The complexes may be used for the preparation of a vaccine.

In a preferred embodiment, the non-dendritic peptide carrier according to the invention is non-cyclic.

As mentioned above, the present invention relates to a method for preparing a non-dendritic peptide carrier comprising an immunogenic substance coupled thereon comprising the steps of

i) synthesizing a non-dendritic peptide carrier as defined in any of the preceding claims by chemical solid-phase synthesis on a linker, and

ii) synthesizing the immunogenic substance directly on the non-dendritic peptide carrier, and

iii) cleaving the non-dendritic peptide carrier from the solid phase. In addition, step ii) may further comprise covalent attaching other moieties to the peptide carrier by use of derivatizable groups thereon.

The non-dendritic peptide carrier according to the invention may also be used as a scaffold for the production of chemical derivatives, characterised by covalently attached molecules at the attachment points, the molecules being selected from peptides, carbohydrates, haptens, glycopeptides, lipopeptides, DNA, RNA, PNA, proteins and glycoproteins and combinations thereof. In addition, the scaffold-peptide complex may be incorporated into Immunostimulating Complexes (Iscoms) by use of a lipidic moiety resulting in a non-dendritic peptide carrier-Iscom complex. Two or more different complexes may be incorporated into the same Iscom.

The non-dendritic peptide carrier as defined above is suitable as a diagnostic component for detecting a molecule or a substance as one or more diagnostic agents, e.g an antigen or antibody such as a polypeptide, lipopolypeptide, a glycopolypeptide, a phospholipid, a carbohydrate, a lipopolysaccharide or a nucleotide sequence (including a DNA sequence, an RNA sequence, or any modification thereof), PNA or any combinations or modifications thereof which may be linked to the carrier. In one embodiment, the diagnostic component may comprise at least two different diagnostic agents capable of detecting the same or different molecules.

Such a diagnostic component may be used use in vivo directly in an animal or in vitro by use of a sample.

The diagnostic agent is used in an amount which is effective to detectably react with said molecule to be detected and to which the diagnostic agent is capable of binding. The diagnostic component is used for detection a molecule by incubating the diagnostic component with the molecule for a time sufficient for the diagnostic component, e.g. in a suitable

composition to react with the subject and forming a complex and detecting the presence of bound molecule by subjecting said complex to a detecting means.

The diagnostic component be used according to the invention for detection of a molecule derived from or indicative of pregnancy, of a disease, such as an infectious disease, an autoimmune disease, a cancerous disease or any other disease wherein an indicative molecule is known, e.g by use of a sample derived from tissue including a biopsy or tissue extract, a cell culture or the animal, including a human being. Accordingly, the sample may be derived from serum, plasma, whole blood, cerebrospinal fluid, seminal or vaginal fluids, exudates, saliva, urine, faeces, or the like.

In one embodiment, the diagnostic component is administered directly to the aminal in which the diagnostic agent is complementary to a molecule present in the animal and indicative of the disease or pregnancy of said animal.

In a further use according to the invention, the derivatized non-dendritic peptide carriers according to the invention is used for the selection of specifically peptide binding oligonucleotides, especially oligoribonucleotides ("aptamers"). Such aptamers have been shown to be able to bind specifically and with high affinity to specific peptides in an induced-fit mode resembling antibody binding (Xu 1996). Anti-peptide aptamers are of interest as an alternative to antibodies in diagnostic assays and in therapy based on binding to and blocking of e.g. virus-derived mRNA-binding proteins. Aptamers are selected in an *in vitro* process from a large pool of random oligonucleotides by their binding to the solid-phase coupled peptide. By using the derivatized non-dendritic peptide carriers which are objects of this invention, it is believed that better and more specifically binding aptamers can be selected as the peptide is presented in a more well-defined and structure-supported way. Also, in the present

invention, the selection procedure itself is simplified as the solid-phase coupled complex is used directly.

As explained above, in a further embodiment, the present invention relates to a vaccine component comprising a non-dendritic peptide carrier as defined above on which at least one immunogenic agent or mediator is attached. The immunogenic agent may be a polypeptide, a glycopeptide, a lipopeptide, a phospholipid, a polysaccharide, a lipopolysaccaride, a carbohydrate, a nucleotide sequence, PNA or any combination or modifications thereof. Furthermore, at least one mediator capable of affecting the immunogenic effect of the vaccine component or the reaction of the immune system exposed to the vaccine component may also be linked or form part of the peptide carrier.

Examples of such mediators includes a tuftsin, an immunomodulator including a cytokine such as an interleukin or interferon, an enhancer, or a part or modification of the above having mediator activity. The mediator may be naturally, synthetically or recombinantly derived.

In another embodiment, the vaccine component is attached to a second carrier, such as an Immunostimulating Complex (Iscom) or liposome, optionally in combination with a mediator or a part thereof having mediator activity and attached to the non-dendritic peptide carrier and/or second carrier.

The non-dendritic peptide carrier of this invention may also be used with other secondary carriers, including proteinoid microspheres constituted of derivatised alpha-amino acids (Haas 1996) and poly(lactide-coglycolide) polymer slow release biodegradable microparticles (PLG)(Ertl 1996). Proteinoid microspheres are prepared by derivatized alpha-amino acids that spontaneously form microspheres at low pH and encapsulate antigens by simple mixing of the amino acids with the antigen followed by acidification (Haas 1996). With PLG, an emulsion of the PLG and the antigen is formed fol-

lowed by lyophilization (Ertl 1996). The particles preferably have diameters from 0.5 to 10 μm, preferably from 0.5 to 2 μm and in both cases are biodegradable.

Such particulate formulations have the advantage that they may be administered orally and are immunogenic by oral administration.

The invention also relates to a vaccine composition comprising at least one vaccine component as defined above. The composition preferable comprises an effective amount of the vaccine component to confer increased resistance to one or more infection(s) in the animal, the composition optionally further comprising a pharmaceutically acceptable carrier or vehicle, enhancers or adjuvants.

An example of the use of an adjuvant is the combination of the non-dendritic carrier peptide combined with Schiff-base-forming components which are known to act like adjuvants and enhance the Schiff-base formation between carbonyl-groups and amino groups on antigen-presenting cells and T-cells occuring during a natural response (Gao 1990). This can be done by including a Schiff-base forming substance in the vaccine formulation directly activating T-cells by binding to amino-groups on their surface (Rhodes 1995a) or by administering simultaneously with the immunogenic sustance the two carbohydrate-modifying enzymes neauraminidase and galactose oxidase ("NAGO" adjuvant) (Rhodes 1995b). In a preferred embodiment, a Schiff-base forming aldehyde, as e.g. tucaresol (4(2-formyl-3-hydroxyphenoxymethyl)benzoic acid) is bound as an auxiliary moiety in the non-dendritic peptide carrier. This can be achieved by coupling to a selectively deprotected amino-group in the non-dendritic peptide carrier through activation of the carboxylic acid in tucaresol.

The vaccine composition according to the invention may be used for immunizing an animal including an human being by

administering the vaccine composition nasally, subcutaneously, intramuscularly or by any other convenient route.

In a still further aspect, the present invention relates to a therapeutic component comprising a non-dendritic peptide carrier as defined above to which at least one therapeutic or prophylactic agent is attached, e.g as defined for the vaccine component above. A therapeutic component according to the invention may further comprise at least one mediator, e.g as defined for the vaccine above, capable of controlling or enhancing the effect of the therapeutic or prophylactic agent linked to the carrier.

As described above for the vaccine, the therapeutic component according to the invention may also comprise a second carrier, such as an Immunostimulating Complex (Iscom) or liposome, optionally in combination with a mediator or a part thereof having mediator activity and attached to the non-dendritic peptide carrier and/or second carrier.

In a preferred embodiment of the invention, a therapeutic component according to the invention may also comprise a targeting molecule capable of binding to a target substance present at a specific location in the animal thereby directing the therapeutic component to said specific location where the therapeutic component is to exert its effect. Such targeting molecule is e.g an antibody. Accordingly, the therapeutic component may be capable of preventing, including preventing relapse, or treating a disease or capable of preventing or disrupting pregnancy in an very effective way. Any disease wherein a target substance is identified may be treated according to the invention. Thus, diseases to which the invention is applicable include infectious diseases, cancerous diseases and autoimmune diseases.

The pharmaceutical component may be used in a composition comprising the therapeutic component together with a pharmaceutically acceptable carrier.

Accordingly, the present invention also relates to a method of treatment and/or prevention of a disease, comprising administering to a patient in need thereof a therapeutically or prophylactically effective amount of a pharmaceutical composition described above.

Finally, the present invention also relates to a detecting component for detecting a molecule or a substance, the component comprising a non-dendritic peptide carrier as defined above to which a detecting agent is linked.

Any range or value given herein may be extended or altered without losing the effects sought, as will be apparent to the skilled person for an understanding of the teachings herein.

DESCRIPTION OF FIGURES

Fig. 1A shows a non-dendritic peptide carrier-solid phase complex before attachment of an immunogenic substance.

Fig. 1B shows another embodiment of a non-dendritic peptide carrier-solid phase complex in which an auxiliary segment has been attached.

Fig. 1C shows a branched peptide complex consisting of a non-dendritic peptide carrier-solid phase complex on which immunogenic branch peptides have been attached.

Fig. 1D shows a similar branched peptide complex as shown in Fig. 1C which in addition comprises an auxiliary segment coupled to a double attachment point.

Fig. 1E shows a similar branched peptide complex as shown in Fig 1C which but including an auxiliary segment coupled in a linear arrangement in the branch peptide.

Fig. 2 shows HPLC-analysis of a backbone-peptide before (A) and after (B) coupling of palmitic acid (raw products) (see Example 1).

Fig. 3 shows Matrix-assisted Laser desorption time-of-flight mass spectrometry analysis of palmitoylated backbone-peptide (HPLC-purified) prepared as described in Example 1, using a Fisons linear mode mass spectrometer, and alpha-cyano 4-hydroxy cinnamic acid as the matrix. The results are calibrated and centroid adjusted.

Fig. 4A and 4B show two dibranching linkers A and B respectively.

Fig. 4C shows the structure of the tripalmitate moiety, N-palmitoyl-S-[2,3-bis (palmitoyl-oxy)-(2RS)]-propyl-[R]-cysteinyl.

Fig. 4D shows a glycolic acid linker.

Fig. 5 shows antibody reactivity of a HIV-1 seropositive donor against dilution curves of peptide, gp41 (aa598-609), being tested alone and linked, respectively, to the non-dendritic peptide carrier as described in Example 19.

Fig. 6 shows dilution curves of sera obtained from HIV-2 seropositive donors (———) and HIV-2 seronegative donors (----) tested against the non-dendritic peptide carrier construct. Positive signals were obtained at all dilutions of HIV-2 seropositive sera tested while the seronegative control sera were negative in all the dilutions tested. (see Example 19)

Fig. 7 shows the results from fixed dilutions of sera obtained from a panel of HIV-2 seropositive and HIV-2 seronegative donors tested against the peptide coupled to a non-dendritic peptide carrier and alone (see Example 19). The sera were also tested against recombinant protein HIV-2 gp36.

All the seropositive donorsera were reactive in the assay while no reactivity was detectable with the seronegative control sera.

Fig. 8 shows dilution curves of sera obtained from one HIV-2 seropositive donor and one HIV-2 seronegative donor tested against the gp36 peptide alone and coupled to a non-dendritic peptide carrier. The sera were also tested against recombinant protein HIV-2 gp36. The peptides, when coupled to the non-dendritic peptide carrier, were recognized at higher dilutions of the seropositive serum compared to the peptide alone, see Example 19.

Fig. 9 shows that mice produced antibodies in response to the derivatized non-dendritic peptide carrier EBA peptide-PPD conjugate after immunization by antibody production as described in Example 9. The strongest antibody response was detectable after 3 immunizations. Absorption to aluminium hydroxide enhanced antibody production after one immunization but not after two or three immunizations. (see Example 20)

Fig. 10 shows that mice responded to the EBA peptide after immunization with the derivatized non-dendritic peptide carrier EBA peptide alone intraperitoneally or after immunization with the derivatized non-dendritic peptide carrier EBA peptide mixed with Freund's adjuvant subcutaneously. Levels of antibody reactivities were higher when mixing the branched peptide construct with Freund's adjuvant. (See Example 20)

Fig. 11 shows that mice responded to the gp120 peptide after immunization with this peptide derivatized to the non-dendritic peptide carrier and mixed with Freund's adjuvant subcutaneously. The strongest antibody response was detectable after 3 immunizations. (see Example 20)

Fig. 12 shows percentage inhibition of IL-6 secretion from human mononuclear cells stimulated with antigens from the malaria parasite *Plasmodium falciparum* : (———) inhibition

by the beta-2-glycoprotein I peptide coupled to the non-dendritic peptide carrier; (-----) inhibition by the peptide alone. Means of 2 different experiments are shown. (see Example 22)

Fig. 13 shows HPLC analysis of a non-dendritic peptide carrier structure type 2 (see Table 1) derivatized with HIV gp120-peptide. HPLC-analysis of raw product at 280 nm (top) and 220 nm (bottom). "X" is the injection peak, and "Y" is a system peak. "P" is the desired product peak.

Fig. 14 shows that mice produced IgG1 in response to subcutaneous immunization with the EBA peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant.

Fig. 15 shows that mice produced IgG2a in response to subcutaneous immunization with the EBA peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant.

Fig. 16 shows that mice produced IgG to the LERLLL HIV-1 gp41 peptide in response to subcutaneous immunization with the LERLLL HIV-1 peptide derivatized non-dendritic peptide carrier mixed with HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier peptide with and without Freund's complete adjuvant.

Fig. 17 shows that mice produced IgG to recombinant HIV-1 gp120 in response to subcutaneous immunization with the HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant.

Fig. 18 shows that mice produced IgG to recombinant HIV-1 gp41 in response to subcutaneous immunization with the LERLLL HIV-1 peptide derivatized non-dendritic peptide carrier mixed with HIV-1 gp120 (aa152-176) peptide derivatized non-

dendritic peptide carrier peptide with Freund's complete adjuvant.

Fig. 19 shows that mice produced IgG1 to HIV-1 gp120 (aa152-176) peptide in response to subcutaneous immunization with the HIV-1 (aa152-176) peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant.

Fig. 20 shows that mice produced IgG2a to HIV-1 gp120 (aa152-176) peptide in response to subcutaneous immunization with the HIV-1 (aa152-176) peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant.

Fig. 21 shows that mice produced IgG1 to the LERLLL HIV-1 gp41 peptide in response to subcutaneous immunization with the LERLLL HIV-1 peptide derivatized non-dendritic peptide carrier mixed with HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier peptide.

Fig. 22 shows that mice produced IgG2a to the LERLLL HIV-1 gp41 peptide in response to subcutaneous immunization with the LERLLL HIV-1 peptide derivatized non-dendritic peptide carrier mixed with HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier peptide.

Fig. 23 shows inhibition of TNF secretion in vivo stimulated by LPS by CKNKEKKC- and KNGMLKGDKVS-derivatized non-dendritic peptide carriers.

Fig. 24 shows that mice produced IgG in response to three subcutaneous immunizations with the EBA peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant but that the IgG response was diminished by a fourth immunization with the EBA peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant and murine recombinant IL-10 compared to a fourth immunization with the EBA peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant alone.

Fig. 25 shows that mice produced IgG1 to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier mixed with different murine recombinant cytokines or with alumn or with Freund's complete adjuvant.

Fig. 26 shows that mice produced IgG2a to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with murine recombinant TNF or with Freund's complete adjuvant.

Fig. 27 shows that mice produced IgG1 to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with tuftsin or with Freund's complete adjuvant.

Fig. 28 shows that mice produced no IgG2a to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with tuftsin.

Fig. 29 shows that mice produced IgG1 to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with different gamma-interferon specific peptides or with Freund's complete adjuvant.

Fig. 30 shows that mice produced IgG2a to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with different gamma-interferon specific peptides or with Freund's complete adjuvant.

Fig. 31 shows that mice produced IgG1 to the L1 leishmania peptide in response to intraperitoneal immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with different gamma-interferon specific peptides.

Fig. 32 shows that mice produced no IgG2a to the L1 leishmania peptide in response to intraperitoneal immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with different gamma-interferon specific peptides.

Fig. 33 shows that mice produced IgG1 to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with recombinant TNF or a TNF specific peptide or with Freund's complete adjuvant.

Fig. 34 shows that mice produced IgG2a to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with recombinant TNF or with Freund's complete adjuvant.

Fig. 35 shows that mice produced IgG1 to the L2 leishmania peptide in response to subcutaneous immunization with the L2 leishmania peptide derivatized non-dendritic peptide carrier with or without a TNF specific peptide or with and without tuftsin.

Fig. 36 shows that mice produced IgG2a to the L2 leishmania peptide in response to one single subcutaneous immunization with the L2 leishmania peptide derivatized non-dendritic peptide carrier with a TNF specific peptide.

Fig. 37 shows that mice produced IgG1 to the HIV-1 gp120 (aa152-176) peptide in response to subcutaneous immunization with the HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier with a TNF specific peptide or with gamma-interferon specific peptides or with tuftsin.

Fig. 38 shows that mice produced IgG2a to the HIV-1 gp120 (aa152-176) peptide in response to one and three subcutaneous immunizations with the HIV-1 gp120 (aa152-176) peptide deriv-

atized non-dendritic peptide carrier with a TNF specific peptide or with gamma-interferon specific peptides.

Fig. 39 shows that mice produced IgG to the HIV-1 gp120 (aa152-176) peptide in response to subcutaneous immunization with the HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier with or without an IL-1 specific peptide or tuftsin or mixed with Freund's complete adjuvant.

Fig. 40 shows that mice produced IgG to the HIV-1 gp120 (aa152-176) peptide in response to subcutaneous immunization with the HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier with or without an IL-1 specific peptide.

Fig. 41 shows the development of antibodies against Tbp-peptide after immunizations with peptide conjugated to PPD and with the peptide coupled to a non-dendritic peptide carrier (NDPC) with and without use of adjuvant. The analysis was performed by ELISA.

Fig. 42 shows the development of antibodies against PalA-peptide after immunizations with peptide conjugated to PPD and with the peptide coupled to a non-dendritic peptide carrier (NDPC). The non-coupled, linear peptide was used as an immunization control. The analysis was performed by ELISA.

Fig. 43 shows the reactivity of different constructs (backbone structure numbers refer to Table 1) with a monoclonal antibody raised against PPD-coupled Tbp-peptide 4. The different constructs were used for coating of the microtiter plate and then probed with the monoclonal antibody. The analysis was performed by ELISA.

EXAMPLES

In the examples below, the following abbreviations have been used:

Standard single-letter and three-letter abbreviations for amino acids.

Aib: alpha amino isobutyric acid.
Alo: allyloxycarbonyl.
BCG: Bacterin Calmette Guerin
Boc: butyloxocarbonyl.
BrAc: bromoacetyl.
CA: citraconic anhydride.
DCC: dicyclohexylcarbodiimide.
DCM: dichloromethane.
Dde: 1-(4,4dimethyl-2,6-dioxocyclohexylidine)ethyl.
DMAP: dimethylaminopyridine.
DMSO: dimethylsulfoxide.
DSS: disuccinimidyl suberate
EBA: erythrocyte binding antigen
EDC: 1-ethyl-3[dimethyl(aminopropyl)]carbodiimide
ELISA: enzyme linked immunosorbent assay
Fmoc: fluorenylmethoxycarbonyl.
HOBt: hydroxybenzotriazole.
HMB: 4-hydroxymethyl benzoic acid.
HMPA: 4-hydroxymethyl phenoxyacetic acid.
IFN: interferon
IL: interleukin
i.p.: intraperitoneally
MSNT: 1-(mesitylene-2-sulfonyl)-3-nitro-1-H-1,2,4-triazole
Mtt: methyltrityl.
NMM: N-methylmorpholine.
NMP: N-methylpyrrolidone.
Palm: palmitoyl residue ($CH_3(CH_2)_{14}CO$-).
Pbf: 2,2,4,6,7-penta-methyldihydrobenzofuran-sulfonyl
PPD: purified protein derivative from *Mycobacterium tuberculosis*
s.c.: subcutaneously
SPDP: N-succinimidyl 3-(2-pyridyldithio) propionate.
TBTU: 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.
TFA: trifluoroacetic acid.

TNF: tumour neurosis factor
Trt: trityl.

Example 1

Amphiphilic parallel α-helix homodimer-forming non-dendritic peptide carrier

Examples of basic chemical strategies

A twenty-amino acid α-N-palmitoylated lipopeptide with a strong α-helix, parallel homodimer-forming tendency was defined based on the heptad-rules detailed above:

palm-AVHKLEHKVAKLEAKGKGKY,

where palm=palmitoyl residue ($CH_3(CH_2)_{14}CO$-). Histidine residues were included to support helix-formation upon divalent metal-ion chelation, and tyrosine was included as a 280 nm reporter group. The peptide corresponds to two turns of an α-helix with a loosely structured hydrophilic C-terminal stretch (GKGKY).

The synthesis was performed with Novasyn KB resin (Novabiochem 01-64-0025) containing the base-labile 4-hydroxymethylbenzoic acid (HMB-) linker for C-terminal attachment of the peptide. All amino acid derivatives were from Novabiochem and all solvents from Merck. The amino acids used were α-amino-Fmoc-protected derivatives with the following side-chain protections: Asn, Cys, Gln, His: trityl (Trt), Lys, Trp: butyloxycarbonyl (Boc), Glu, Asp, Ser, Thr and Tyr: tertbutyl (tBu), Arg: 2,2,4,6,7-pentamethyl dihydrobenzofuran-sulfonyl (Pbf). Peptide synthesis was performed on a Mark-III peptide synthesizer from Schafer-N (Copenhagen) using dedicated software. All operations were performed in a fume hood. The quantitative Kaiser test (Kaiser 1970) was used to detect free amino groups.

Measurement of liberated Fmoc (after piperidine) was done at 301 nm as described by Grant (1992) using the formula $[E_{301} \times \text{dil.} \times \text{vol.}]/[10 \times \text{weight}_{gram}]$ (μmol/g), where dil. and vol. are the dilution and the volume, respectively, of the sample used for measurement. Weight is the weight of the peptide-resin from which the Fmoc-group is derived.

As an example, 1 g Novasyn KB resin (Novabiochem 01-64-0025) containing 0.15 mmol/g hydroxyl groups was left in NMP for 20 minutes at room temperature and then washed on a filter with DCM. The first amino acid (tyrosine) was coupled as an ester (Blankemeyer-Menge 1990): 300 μmol (=2 times molar surplus) Fmoc-Tyr(tBu) was mixed with 225 μmol methylimidazole and 300 μmol 1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazol (MSNT) at 0.2 M in DCM and incubated with the KB-resin in DCM with shaking for 60 minutes at room temperature (RT). This coupling was repeated once with new reagents on the same portion of KB-resin followed by capping with 10 equivalents of acetic anhydride in the presence of 1 eq. DMAP. Capping was done for 15 minutes and followed by extensive wash in NMP. Spectrophotometric determination (301 nm) of the Fmoc-group cleaved from the coupled tyrosine (cleavage by 20% piperidine in NMP) gave 134.8 μmol/g resin, which corresponds to a coupling yield of 89.9%. The Kaiser test performed on a small sample of resin at this point yielded a deep blue colour. The rest of the amino acids after this point were coupled sequentially after activation for 10 minutes (TBTU/-HOBt/NMM, 1/1/2 molar equivalents compared to amino acid in NMP) and coupling for 30 minutes in 8 times molar excess, except isoleucine, threonine, and valine which were double-coupled for 2 x 30 minutes. The final Fmoc-value corresponded to 133.9 μmol peptide pr. g resin (original weight). This corresponds to a yield compared to the first amino acid coupled of 99.3%. The Kaiser test gave a deep blue colour. At this point, a small sample of resin was removed for cleavage of the peptide as a control (peptide before palmitoylation, Fig. 2A). The rest of the peptide was palmitoylated by a two times repeated treatment with palmitate as follows: Palmitic

acid corresponding to 10 times the α-amino groups on the peptide-resin was mixed with TBTU, HOBt and NMM (1/1/2 (mol/-mol/mol) corresponding to the palmitic acid) in NMP and activated by shaking at RT for 10 minutes before incubating with the peptide-resin for 30 minutes at RT. After repeated coupling, the Kaiser test yielded a clear colourless solution and a small sample was retained for analysis (peptide after palmitoylation, Fig. 2B). Subsequently, the peptide resin was washed with NMP, 2-propanol, and DCM. To prepare the lipopeptide-resin for attachment of side-chain antigens ("branches"), ε-amino-protecting Boc-groups in lysine were removed by treatment with 6 × 5 ml 50% TFA in DCM for 6 × 30 minutes in a closed bottle with shaking at RT. This was followed by washing the resin on a filter with DCM, 4% NMM in NMP, NMP, water, and ether. At this stage, the peptide-resin was ready for coupling of branch-antigens (see examples below), and the Kaiser test was deep blue. Coupling of a protected amino acid to these attachment points was performed by the normal activation chemistry and yielded a clear peptide-resin by the Kaiser test.

To analyse for the success of synthesis, the two peptide-resin samples (the non-palmitoylated sample and the finished lipopeptide-resin) were treated to remove side-chain protecting groups as above and then cleaved from the resin in the following way:

The peptide resin (100 mg) was treated with 5 ml 1 M NaOH for 10 minutes on ice in an ultrasonic water bath. The resin was then filtered off and the filtrate was collected in 0.1 M acetic acid on ice. This was repeated and followed by 2 times water (MilliQ) incubations for 2 × 10 minutes and 100% acetonitrile in the same way, collecting all filtrates separately in acetic acid. These fractions were then analysed for peptide-yield and purity by HPLC, and relevant fractions were pooled, desalted by preparative reverse-phase HPLC (see Example 5), and freeze-dried. The non-palmitoylated peptide

was predominantly found in the first NaOH-wash, while the palmitoylated peptide was found in both NaOH-washes.

The products were highly soluble in aqueous solution and were easily analysed with good purities (>80%) by HPLC. HPLC-analysis: Solvent A: 0.1% TFA/10% acetonitrile in milliQ water, solvent B: 0.1% TFA in acetonitrile. Flow: 1 ml/min. Column: Merck Lichrospher 100, RP 18, 5 µm, 250 x 4 mm. The gradient was controlled by high-pressure side two-pump mixing (Jasco PU880 pumps) and ran from 100% A at t=0 min. to 20% A at t=10 min. and 0% A at t=20 min. to 25 min. followed by a rise to 100% A at t=26 min. The effluent was monitored at 220 nm on a Varian 9050 detector. Data were collected by Varian Star chromatography software. Load: 50 µl 1 mg/ml, freeze-dried, desalted raw-product pool. The main impurities in the palmitoylated peptide derived from incomplete palmitoylation. The palmitate-coupled non-dendritic peptide elutes considerably later than the non-palmitoylated control-peptide (in this HPLC-gradient around 5 minutes later, the HPLC-analysis is disclosed in Fig. 2). Typical raw product yields were around 80% and desalting yields also around 80%.

Matrix-assisted Laser-desorption ionization time-of-flight mass spectrometry of the products yielded a molecular ion with a mass corresponding well to the expected mass (within 2 D from the expected mass of 2473.1 for the palmitoylated product, see Fig. 3). Various analyses for the secondary structure and the aggregation state of the non-dendritic peptide, including chelation of Zn++- and Co++-ions and spectrocopy, circular dichroism, tyrosine fluorescence quenching, and gel filtration, are to be performed.

The non-dendritic peptide derivatized resin was washed, dried, and stored and used directly for standard peptide synthesis of branch peptides or used for coupling of presynthesized peptides or other whole molecules.

An equally useful peptide was obtained by replacing the double-histidine chelating site using A at these positions.

In another synthesis, I will be used instead of V.

As an alternative to cleavage by NaOH, methanolic ammonia was also employed as this treatment yields the preferred peptide amide.

Also, the peptide will be synthesized using the modified Rink linker (4-(2',4'-dimethoxyphenyl Fmoc-aminomethyl)phenoxy acetic acid) and the amino acid derivatives listed above. In this strategy, Boc will be removed from K by 25% TFA in DCM by a 3 x 10 minutes treatment. This does not affect the binding to the modified Rink linker. This leads to a final, preferred peptide amide product. Also, the cleavage and work-up procedure is simpler than with the 4-hydroxymethyl benzoic acid linker described above. Finally, the modified Rink-linker is stable to hydrazine, allowing extensive use of Dde-protection (see below).

The backbone peptide will also be synthesized with orthogonally protected lysine residues, e.g. Fmoc-K(Mtt)-OH (Mtt=methyltrityl), Fmoc-K(Dde)-OH (Dde=1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-ethyl), Fmoc-K(Aloc)-OH (Aloc=allyloxo-carbonyl), in which side chain protecting groups are removable by 1% TFA, 2% hydrazine and Pd(0)(catalytic hydrogenation), respectively. After synthesis, alpha-amino lipid-coupling and selective lysine-side chain demasking, synthesis or coupling of branch peptides or moieties can then be accomplished with no risk of interference from unmasked functional groups in the side chains of other amino acids in the backbone peptide, especially the carboxylic acid deriving from E.

The change to a selectively cleavable protecting group for side-chain amino groups decreases the heterogeneity in the raw product of the final side-chain derivatized non-dendritic peptide carrier (see Example 5), because of the exclusion of

the possibility of lactam-formation between free side-chain carboxylic groups being activated by TBTU/HOBt/NMM from the branch peptide synthesis and reacting with the deprotected amino groups of the peptide carrier and of the side-chain peptide being synthesized. Also, selective chemistry allows the deprotection of only a part of the lysine epsilon amino groups present, retaining the charge on the lysines taking part in the stabilization of the amphipathic helix (Table 1, structure nos. 2, 3, 5, 6, 7, see below) and/or allowing the inclusion of stimulatory peptides not to be side-chain derivatized (Fig. 1, structure nos. 4 and 5).

As a particular illustrative and preferred example of a combination of linker and protecting group strategy employing selective chemistry, a non-dendritic peptide carrier containing only a single class of attachment sites was synthesized on the acid-labile modified Rink-linker in combination with either hyper acid-labile side-chain amino-protecting groups (methyltrityl- (Mtt-) group, structure nos. 3 and 6) or the hydrazine-labile Dde-group (1-(4,4-dimethyl 2,6-dioxocyclohexylidine)-ethyl)(structures 2, 4, 5). The non-dendritic peptide carriers depicted in Table 1, structure nos. 2-7 given below, were synthesized using the following conditions:

All conditions, solvents and reagents as above, except Fmoc-Lys(Dde)-OH and Fmoc-Lys(Mtt)-OH, which were from Novabiochem (nos. 04-12-1121 and 04-12-1137, respectively). For the coupling of the first (C-terminal) amino acid, the modified Rink-resin (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl) phenoxyacetamido-norleucyl methylbenzhydrylamine polystyrene), Novabiochem 01-64-0037) was treated with 20% piperidine in NMP for 2 x 5 minutes, washed with NMP and coupled with the amino acid in a molar surplus of 8 times using TBTU/HOBt/NMM activation for 10 minutes and coupling for 30 minutes at RT. After a second coupling using the same conditions, the coupling yields were typically from 80-90%. After capping with acetic anhydride/DMAP in NMP (20 equivalents of acetic anhydride, 1 equivalent of tertiary amine (DMAP or NMM), 10

minutes) followed by thorough washing by NMP automated synthesis was performed as above. After cleavage of the final Fmoc-group, palmitic acid was introduced as above and, subsequently, lysine side-chain protecting groups were removed:

Mtt: 1% TFA in 5% triisopropylsilane in DCM, 3 x 5 minutes treatment. Liberated Mtt was monitored at 470 nm.

Dde: 2% hydrazine in NMP, 3 x 5 minutes, liberated Dde was monitored at 290 nm.

The yield of side-chain attachment points were generally from 60-80 %.

After this, to analyse for the purity of the non-dendritic peptide carriers, a small portion of the peptide-resins was cleaved by the standard TFA-cleavage procedure (95 % TFA in water, no scavengers added, 3 x 30 minutes treatment in closed container, followed by precipitation in cold diethyl-ether, filtration, dissolution in water and freeze-drying) and analysed by analytical reverse-phase HPLC.
Peptides were of good purities and yields, and mass spectrometry of the main raw product peak is expected to give molecular weight values not differing more than 5 Da from the theoretical molecular weights given in Table 1.

Synthesized structures

The two types of model structures depicted in Table 2 have been synthesized with Mtt-protection to verify structure by MS-analysis. Mtt was removed by 1% TFA/5% triisopropylsilane in DCM, and a branch-peptide (YLEN and WNSG, respectively) was synthesized on half of each backbone. After cleavage and purification, all constructs will be analysed by MALDI-TOF mass spectrometry. The mass spectrometry analysis is expected to give molecular weights not differing more than 5 Da from the theoretical values depicted in Table 2.

A number of heptad-containing model non-dendritic peptide carriers will also be synthesized, in which two types of protections for lysine were employed; a selectively cleavable one for attachment-point lysines, which includes only lysines at the "c"-positions in the heptads and Boc-protected lysines at the "g"-positions. This allows derivatization at the "c"-positions and, at the same time, retention of charge at the "g"-positions. Also, another design will be used in which an additional selectively deblockable K-residue will be introduced at the f-positions. Thus, the following model peptides will synthesized with the selectively deblockable lysine residues being denoted K:

VAKLEAKVAKLEAK (1)
VAKLEAKVAKLEAK (2)
VAKLEKKVAKLEKK (3)

These syntheses will be performed on a modified Rink-linker as described above. After deblocking the K-residues, they will be derivatized with AELGGQFHHKSENG, cleaved, and worked up and analysed for alpha-helix content as well as for homodimer formation compared to the unsubstituted model peptide. Accessibility and antigenicity of the attached peptide will be analysed with a monoclonal antibody raised against the peptide.

Helix- as well as homodimer-formation is expected to decrease in the derivatized peptide (2) compared to the two other peptides, which are expected to be good alpha-helix- and dimer-formers even after side-chain derivatisation. Also, peptides (1) and (3) are expected to present the side-chain peptides more efficiently to the monoclonal antibody, resulting in a more efficient recognition in an indirect ELISA than peptide 2.

Other variants

By combining three different side-chain protection labilities (weak acid, stronger acid, and hydrazine), it was possible to

synthesize non-dendritic peptide carriers employing both "normal" side-chain attachment points as well as special attachment points for coupling stimulatory peptides, e.g. cytokine peptides (see Example 5) (see Table 1, structure 7).

A range of other standard peptide carrier variants will also be synthesized and analysed in a similar way; these variants include a peptide carrier that is lactam-stabilized by substituting the histidine pair with a lysine-glutamic acid pair and, subsequently, allowing their side-chains to react by selectively deprotecting the side-chains; another variant is substituted with secondary structure nucleators at the attachment points; yet another variant will be synthesized on a dibranched linker molecule, yielding a non-dendritic peptide consisting of two peptide chains held together at their C-terminal; also, a variant with the tripalmitate structure of Fig. 3 instead of a linear palmitate will be synthesized.

Finally, a non-dendritic carrier peptide in which the palmitate is attached by a thioester linkage to the side-chain thiol group in a N-terminal cysteine will be synthesized and used for immunization experiments.

Table 1	
Types of non-dendritic peptide carriers. Molecular weights (mw) are given for the unprotected peptides.	
1	Palm-AVH***K***LEH***K***VA***K***LEA***K***G***K***G***K***Y-OH (***K***=K(Boc)), mw.:2473.1
2	Palm-AVH***K***LEHKVA***K***LEAKG***K***G***K***Y-NH_2 (***K***=K(Dde)), mw.:2472.1
3	Palm-AVH***K***LEHKVA***K***LEAKG***K***G***K***Y-NH_2 (***K***=K(Mtt)), mw.:2472.1
4	Palm-AVH***K***LEH***K***VA***K***LEA***K***G***K***G***K***YVQGEESNDK-NH_2 (***K***=K(Dde)), mw.:3459.1
5	Palm-AVH***K***LEHKVA***K***LEAKG***K***G***K***YVQGEESNDK-NH_2 (***K***=K(Dde)), mw.: 3459.1
6	Palm-AVA***K***LEAKVA***K***LEAKG***K***G***K***Y-NH_2 (***K***=K(Mtt)), mw.:2340.0
7	Palm-***K***VA***K***LEAKVA***K***LEAKG***K***G***K***Y-NH_2 (***K***=K(Dde) and ***K***=K(Mtt)), mw.:2397.1

Table 2	
Types of model non-dendritic peptide carriers	
1	***K***G***K***G***K***GL-NH2 palm-***K***G***K***G***K***GL-NH2
2	***K***GKG***K***GL-NH2 palm-***K***GKG***K***GL-NH2
3	KG***K***G***K***GL-NH2 palm-KG***K***G***K***GL-NH2
	K=Lys(Boc) ***K***=Lys(Mtt). palm=palmitoyl residue Molecular weights (mw) are given for the unprotected peptide. Mw.: 685.9 (non-palmitoylated peptides) 924.3 (palmitoylated peptides) Branch-peptides: YLEN- Resulting molecular weights: 1732.9 (2 attachment points, non-palmitoylated) 1971.4 (2 attachment points, palmitoylated) 2256.6 (3 attachment points, non-palmitoylated) 2495.0 (3 attachment points, palmitoylated)
4	GKGKGKLYL, where K=Lys(Mtt) mw (unprotected): 962.2
5	Palm-GKGKGKLYL, where K=Lys(Mtt) mw (unprotected): 1200.6
6	N^{ϵ}[WNSG]$_3$-palm-GKGKGKLYL, where K=Lys(Mtt) mw (unprotected): 2534.0

Example 2

A solid-phase for the simultaneous synthesis but separate release of derivatized non-dendritic peptide carriers as well as free peptides

The following method was devised for the purpose of obtaining, directly by solid-phase synthesis, both a non-dendritic peptide carrier (NDPC) coupled peptide and the same peptide in a free form that allows direct inspection of the success of the synthesis and gives access to a control-peptide for immunization experiments, for characterization of antibodies etc.

This can be achieved by substituting a solid-phase resin with a mixture of orthogonally labile linkers, e.g. an acid-labile linker as 4-hydroxymethylphenoxy acetic acid (HMPA) combined with a base-labile 4-hydroxymethylbenzoic acid (HMB). Then one of the linkers is blocked temporarily, while a non-dendritic peptide carrier (NDPC) is synthesized on the other linker. The treatment that removes NDPC side-chain protecting groups also removes the protecting group on the other linker. In an example of such a synthesis, a Novasyn base resin will be partially substituted by the HMPA-linker by incubation of the resin with TBTU/HOBt/NMM-activated linker in a 0.1 molar ratio to the number of amino groups on the resin, coupling in NMP for 1 x 30 minutes at room temperature under shaking. Then the resin will be washed with NMP, and the remaining amino groups will be blocked by a 5 times molar surplus of Fmoc-norleucine activated as above for 2 x 30 minutes at room temperature with shaking. Following a wash in NMP, Boc-glycine will be coupled to the free hydroxyl-group of the HMPA-linker by a mesitylene/imidazole coupling (see Example 1). After wash with NMP, the α-amino group of the Fmoc-L will be removed by 20% piperidine in NMP. After yet another NMP-wash, the HMB-linker will be attached by activation and coupling twice as described above with 5 times molar surplus. The NDPC will be synthesized on this solid phase as detailed

in Example 1. Side-chain protecting groups as well as the Boc-G coupled to the HMPA-linker will be cleaved by 95% TFA in water. HMPA is now ready for coupling another amino acid as an ester. The resulting solid phase can be used for the simultaneous synthesis of the free peptide and the peptide-derivatized NDPC, the former being liberated and deprotected in one step by TFA-treatment (95% TFA in water), while the derivatized NDPC is only released by base-treatment (see Example 1).

In another synthesis to be performed, the resin will be partially substituted by a Fmoc-protected RINK-linker by incubating the resin with TBTU/HOBt/NMM-activated linker in a 0.1 molar ratio to the number of amino groups on the resin, coupling in NMP for 30 minutes (once) at room temperature with shaking. Subsequently, the HMB-linker will be coupled by activation as above to the rest of the available amino groups on the resin coupling twice (30 minutes, room temperature, shaking) with 5 times molar surplus. Now the Fmoc-group is removed from the Rink-linker by 20% piperidine/NMP, and a Boc-G is coupled to the free amino group by TBTU/HOBt/NMM activation chemistry. These coupling conditions favour coupling to the amino groups compared to esterification to the hydroxyl-groups of the HMB-linker. The NDPC is now synthesized on this solid phase as shown in Example 1. In the deprotection step with 50% TFA in DCM, the Boc-groups protecting the ϵ-amino group of the backbone lysines as well as the Boc-group of the Rink-linker-bound G will be removed. The resulting solid phase can be used for the simultaneous synthesis of the free peptide and the peptide-derivatized NDPC, the former being liberated and deprotected by extensive TFA-treatment (95% TFA in water), while the derivatized NDPC is only released by base-treatment (see Example 1). The free peptide will be extended in the carboxylic end by one glycine residue.

Example 3

Non-dendritic peptide building blocks for creating looped peptides

Loops are very common and easily distinguishable features of most proteins of which the tertiary structure is known. Moreover, such loops often constitute immunodominant parts of the protein, and a method for their easy and general preparation in a loop-inducing set-up is therefore highly interesting. As an example, this can be done by synthesizing a first non-dendritic building block, palmitoylated tetrapeptide PC(Trt)K(Palm)L, on a Novasyn KB resin by the solid-phase method detailed in Example 1. The Fmoc-group will be left on K, and the ϵ-amino group will be deprotected by treatment with 50% TFA in DCM as in Example 1. Subsequently, palmitic acid will be coupled to this amino group as described in Example 1, the completeness of coupling being followed by the Kaiser test. Hereafter, the α-amino Fmoc-group will be cleaved and the rest of the peptide will be synthesized. The peptide will be left on the resin after cleavage of the last Fmoc-group. The other building block could be an identical peptide but soluble and cleaved from the resin by NaOH-treatment as described in Example 1, but not deprotected by acid and still containing the last Fmoc-group. As another example of the soluble building block, Palm-PC(Trt)LG will also be synthesized. As yet another example of this soluble building block, Palm-PK(BrAc)LB will be synthesized, in which BrAc is a bromoacetyl moiety introduced by reacting the ϵ-amino group of the K residue with bromoacetic acid after side-chain deprotection with TFA/DCM. The target-peptide to be presented as a loop (target peptides to be included will be peptides corresponding to loops in human IL-1β (LQGQDMEQQV (aa31-40 in the circulating form of the protein), DPKNYPKKKMEKRF (aa86-99 in the circulating form of the protein), VQGEESNDK (aa47-55 in the circulating form of the protein), GGTKGGQDIT (aa135-144 in the circulating form of the protein and the correspon-

ding loops in porcine interleukin-1β) are now synthesized on the free alpha-amino group of the solid-phase bound peptide by standard methods (Example 1). In target peptides containing cysteine, target peptide C will be protected orthogonally to C(Trt). After cleavage of the last Fmoc-group of this solid-phase bound peptide, the free building block will be activated in a big molar surplus (10 times) and coupled with TBTU/HOBt/NMM in NMP to the solid-phase bound peptide as described in Example 1 for palmitic acid until the Kaiser test is negative. The Fmoc-group and side-chain protecting groups will be removed as described in Example 1, and cyclization on the resin will be done by oxidation with I_2/NMP until a negative Ellman-reaction (Ellman, 1959). With the bromoacetylated building block, cyclization occurs in PBS, pH 7.2, on the resin with a simultaneous release of HBr, forming a stable thioether. Remaining Cys-protecting groups, if any, will be removed by their specific treatment, after which the whole complex will be liberated by NaOH as above. Disulfide-formation may also be performed in solution after liberation of the linear peptide by using low concentration of the peptide under disulfide-forming conditions (see e.g. Grant 1992). Other functional group pairs may be used in analogy with the methods described above, as long as any potentially interacting groups in the loop peptide are orthogonally protected. Examples to be perfomed include lactam-bridge formation between glutamic acid or aspartic acid and lysine. After synthesis and cleavage, peptides will be analysed on HPLC as described in Example 1. Cyclic peptides are expected to elute before the corresponding linear ones.

Example 4

Anchoring peptide.

The HMB-linker allows the preparation of peptide hydrazides (C-acyl-hydrazines) by cleavage with hydrazine. Peptide hydrazide is an excellent reagent for coupling mildly oxidized or reducing carbohydrates or carbohydrate-containing

compounds, e.g. glycoproteins. An example of the possible synthesis of such a peptide is as follows:

A peptide, such as the ones listed below, will be synthesized as described in Example 1 and derivatized with α-N-terminal palmitic acid before deprotection with 50% hydrazine-hydrate in dioxane/methanol (9/1) for 2 hours at 4°C, followed by filtration, wash in the same solvent and neutralization of the filtrate, evaporation, and finally dissolution in water and lyophilization (NovaBiochem 1994). The peptide will be selected from the following:

- a general T-cell stimulatory peptide such as QYIKANSKFIGITE (tetanus toxoid 830-843), FNNFTVSFWHRVKVSASHLE (tetanus toxoid 947-967), DQVHFQPLPPAVVKLSDALI (*Mycobacterium tuberculosis* 38 kD antigen 350-369), DIEKKIAKMEKASSVFNVVNS (*Plasmodium falciparum* circumsporozoite protein 378-398), KLLSLIKGVIVHRLEGVE (measles virus F-protein 286-302), LDNIKGNVGKMEDYIKKNNK (*Plasmodium falciparum* MSP-1, 260-279), and polyepitope constructs consisting of a linear covalent arrangement of a number of different T-cell epitopes without flanking sequences (see Thomson 1995);
- a generally stimulating peptide such as tuftsin oligomers $[TKPR]_N$, in which N is preferably from 2-5, or muramyl dipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine);
- a spacer peptide, providing detectability by its content of Y, as in palm-YGLAELKG;
- a combination of a peptide derivatized non-dendritic carrier prepared as described in Examples 5 and 6, but liberated by hydrazine to yield the peptide hydrazide, in which the included branch peptides are constituted by any of the T-cell stimulatory peptides mentioned above.

For peptides not containing Y or W, one Y will preferentially be inserted at one of the ends of the peptide. Reversed phase HPLC-analysis of a standard backbone peptide as described in Example 1, compared with an analysis of the same peptide

cleaved as a hydrazide, will presumably show very little difference between the behaviour of these two peptides in HPLC, both with regard to retention time and purity. Mass spectrometry is expected to show the generation of peptides increased by 14 D, corresponding to the substitution of -COOH by $-CONHNH_2$. The hydrazide anchoring peptide will then be desalted, freeze-dried, and used for conjugating a carbohydrate with accessible carbonyl groups either obtained by controlled mild oxidation by periodate or provided by the reducing end of the carbohydrate (see Example 10).

Example 5

Attachment of peptides by direct, sequential chemical solid-phase synthesis on a non-dendritic backbone peptide carrier

The non-dendritic backbone peptide carrier coupled to the solid phase can be used directly for conventional solid-phase peptide synthesis. One way of doing this is to use conventional sequential petide synthesis methodology, using Fmoc-based chemistry on base- or acid-cleavable backbone-peptides of the types described in Example 1. After synthesis of branch-peptides, protecting groups are removed by TFA, in the case of the modified Rink-linker, simultaneously cleaving the derivatized non-dendritic peptide carrier from the solid-phase, and, in the case of the base-cleavable HMB-linker, followed by standard cleavage of the branched peptide complex by aqueous base and work-up by desalting and freeze-drying. As an example of this, a synthesis was performed in the following way:

A Novasyn KB resin derivatized with the backbone lipopeptide of Example 1 (Table 1, structure 1) was estimated to contain a maximum of 6 times the original amino groups as 6 ϵ-amino groups are introduced at each amino group on the resin. 150 mg derivatized resin, corresponding to 100 mg original resin, was used for synthesis using standard Fmoc-chemistry and TBTU/HOBt/NMM preactivation as described in Example 1. After

synthesis, the last Fmoc-group and side-chain protecting groups were removed, and the whole branched peptide complex was cleaved from the solid phase by the standard treatments described in Example 1. Peptide-containing fractions were desalted as a pool by semi-preparative chromatography on a Waters PrepPak 25 × 10 cartridge holder containing a radially compressed Delta-Pak C18 300 Å cartridge at 10 ml/min using two Jasco PU880 pumps and a step-gradient, eluting at 100% acetonitril/TFA, collecting the complete peptide peak and freeze-drying. The freeze-dried material was highly soluble in aqueous buffers. HPLC analysis showed that the branched lipopeptide complex had a lower retention time than the free lipopeptide-backbone alone. Mass spectrometry as well as a calculation of the actual number of branches obtained in the synthesis were performed. The calculation being based on the E_{280} of a known concentration of the branched peptide, compared to its theoretical Y/W-content and molecular weight, will be performed.

As a particularly preferred example, a modified Rink-MBHA-resin was used for the synthesis in combination with the selective chemistries mentioned in Example 1, i.e. Mtt- and/or Dde-protected lysine in addition to standard Boc-protected lysine. The principles of branch-peptide synthesis are as above, the only difference being the simpler TFA-cleavage and work-up procedure (see Example 1) and, of course, that attachment points are created by selective deblocking.

On backbone no. 1 (Table 1), when nothing else is indicated:

Malarial peptides:

TLTKEYEDIVLKSHMNRESDD (EBA peptide)

LKSHMNRESDDGELYDENS (EBA peptide)

Peptides from Ag332 from *Plasmodium falciparum*:

(VTEEI)

$([VTEEI]_2)$

$([VTEEI]_3)$

Leishmania peptides: (on backbone no. 6 (Table 1))

YDQLVTRVVTHEMAHA

EAEEAARLQA

HIV peptides

LERLLL (HIV gp41 peptide)

GEIKNCSFNISTSIRGKVQKEYAFF (HIV gp120 peptide)

GEIKNCSFNISTSIRGKVQKEYAFF on backbone 2, HPLC-analysis in Fig. 13.

GEIKNCSFNISTSIRGKVQKEYAFF on backbone 4

GEIKNCSFNISTSIRGKVQKEYAFF on backbone 5

GEIKNCSFNISTSIRGKVQKEYAFF on backbone 6

WGCSKLICTTAVPWN (HIV gp41 peptide)

LQDQARLNSWGCAFRQVCHT (HIV gp36 peptide)

Actinobacillus pleuropneumoniae peptides

Tbp-2 (Transferin-binding protein type 2)

SGGKGSFDLEDV (peptide 1)

AELGGQFHHKSENG (peptide 4)

PalA (proteoglycan-associated lipoprotein)

GMTAEDLQTRYN (peptide 1)

TEADYAKNRAVLEY (peptide 5)

Peptides from Salmonella induced filament hypothetical protein, selected by hydrophilicity:

CASQRDRFQVHNPHENDA

CKSQSGIEKTTRILHHANISESTQQN

CQATAKMAEEQLTTLHVRSEQQS

More complex structures were synthesized in which the non-dendritic peptide carrier contained two or more different

side-chain protecting groups for lysine ε-amino groups. As an example of the use of a non-dendritic peptide carrier carrying three different types of side-chain protecting groups, structure no. 7 in Table 1 was synthesized. After palmitoylation, side-chain Mtt-groups were removed by 1% TFA/5% TIS in DCM, followed by attachment of branch peptides either by sequential synthesis or by en bloc couplings. The alpha-amino groups of the side-chain peptides were acetylated with acetic anhydride. Hereafter, the Dde-group was removed by 2% hydrazine in NMP and the liberated amino-group was derivatized with SPDP. Finally, this was coupled with a cysteine-extended peptide.

The following more complex structures were synthesized. Numbers refer to the non-dendritic peptide carrier structures in Table 1. Lys(Dde) and Lys (Mtt) denote lysine residues protected by Dde and Mtt respectively during synthesis.

Mw.= Molecular weight

{[N^{ϵ}(IL-1ßpep-C-Cys)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
Structure 7, mw.: 14862,

{[N^{ϵ}(IL-1ßpep-N-Cys)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
Structure 7, mw.: 14862,

{[N^{ϵ}(IFNγ-1)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
N^{α}[Lys(Dde)]-Structure 6, mw.: 18573,

{[N^{ϵ}(IFNγ-2)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
N^{α}[Lys(Dde)]-Structure 6, mw.: 18604,

{[N^{ϵ}(TNFα)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
N^{α}[Lys(Dde)]-Structure 6, mw.: 15231,

{[N^{ϵ}(IFNγ-1)Lys(Dde)][N^{ϵ}(Leishmania 1)Lys(Mtt)]$_4$}-
N^{α}[Lys(Dde)]-Structure 6, mw.: 14594,

{[N^{ϵ}(IFNγ-2)Lys(Dde)][N^{ϵ}(Leishmania 1)Lys(Mtt)]$_4$}-N^{α}[Lys(Dde)]-Structure 6, mw.: 14625,

{[N^{ϵ}(TNFα)Lys(Dde)][N^{ϵ}(Leishmania 1)Lys(Mtt)]$_4$}-N^{α}[Lys(Dde)]-Structure 6, mw.: 11252,

{[N^{ϵ}(IFNγ-1)Lys(Dde)][N^{ϵ}(Leishmania 2)Lys(Mtt)]$_4$}-N^{α}[Lys(Dde)]-Structure 6, mw.: 11462,

{[N^{ϵ}(IFNγ-2)Lys(Dde)][N^{ϵ}(Leishmania 2)Lys(Mtt)]$_4$}-N^{α}[Lys(Dde)]-Structure 6, mw.: 11493,

{[N^{ϵ}(TNFα)Lys(Dde)][N^{ϵ}(Leishmania 2)Lys(Mtt)]$_4$}-N^{α}[Lys(Dde)]-Structure 6, mw.: 8120,

where

IL-1ßpep: VQGEESNDK (I1-1$_{\beta}$(163-171))
IL-1ßpep-C-Cys:VQGEESNDKC
IL-1ßpep-N-Cys:CVQGEESNDK
IFNγ1(1-39): HGTVIESLESLNNYFNSSGIDVEEKSLFLDIWRNWQKDG(C)
IFNγ2(95-133): AKFEVNNPQVARAAFNELIRVVHQLLPESSLRKRKRSRC
TNF-α(70-80): (C)PSTHVLLTHTI
gp120: GEIKNCSFNISTSIRGKVQKEYEAFF
Leishmania 1: YDQLVTRVVTHEMAHA
Leishmania 2: EAEEAARLQA

(C) denotes a cysteine residue, not part of the natural sequence, added for the purpose of coupling through SPDP (see Example 7).

The peptides will be analysed for purity by HPLC and the molecular weight will be analysed by mass spectrometry. The raw products are expected to contain easily identifiable and purifiable main peptide products (>60%) conforming to the molecular weights given above within 5 Da.

As examples of planned syntheses, the following peptides will be synthesized on a number of backbones including the types no. 3, 5, 6, and 7 depicted in Fig. 1:

$[QGPGAP]_4$ (malarial circumsporozoite protein)
GHPLQKTY (band-3 peptide)
LTPLEELYP (band-3 peptide)
KNGMLKGDKVS (β_2-glycoprotein-I peptide)
CKNKEKKC (β_2-glycoprotein-I peptide)
Peptides from human and porcine IL-1ß, selected by hydrophilicity and homology considerations:
See Example 3.

Other backbones will contain auxiliary peptides selected from cytokine peptides and from the promiscous T-cell binding peptides included in the list in example 4. In particular, tuftsin-peptides ($[TKPR]_N$, with N=3-7) and the IL-1ß nonapeptide VQGEESNDK will be included in a number of linear and branched arrangements (in addition to the linear inclusion of the IL-1ß-peptide in peptide structure no. 5 (Table 1)).

In addition, further examples of synthesized peptides are mentioned in the examples below.

All constructs will be analysed by mass spectrometry and reverse phase HPLC for molecular weight and purity. This will also be done for all constructs mentioned in the following examples.

Low loading of backbone peptide

To investigate the influence of the density of the non-dendritic backbone peptide carrier in the solid phase on the performance as a scaffold for coupling branch peptides, two deliberately low-substituted solid-phase complexes were prepared, one at 31 µmol/g (15 minutes coupling of first amino acid) the other at 61 µmol/g (30 minutes coupling of first amino acid), and both compared to the standard substi-

tution of around 130 μmol/g as in Example 1 on a Novasyn KB resin (Novabiochem). In both cases, additional attachment points on the solid phase resin were blocked by acetic anhydride/DMAP before further synthesis. A model peptide GHPLQKTY was coupled to the non-dendritic backbone peptide solid phase complex by direct synthesis as above, and the Kaiser test was performed and the Fmoc value measured at each step throughout synthesis. It was observed that the maximum number of branches were more easily coupled with higher homogeneity with the low-loaded solid-phase complex.

Direct coupling to multiplied attachment points

Multiplied attachment points are introduced by coupling α-Fmoc-K(ε-Fmoc) to the non-dendritic backbone peptide carrier amino groups. A multiplied attachment point may be introduced at any point during synthesis of branch-peptides, including after the first lysine, to provide a dendritic aspect to the attachment point. Coupling concentration of amino acids and coupling reagents should be adjusted according to the increased number of equivalents. Prolonged coupling times may be necessary in order to overcome the increased "crowdedness" of the backbone.

In the present example, a two turn α-helical amphipathic peptide containing Y [VYKLEAKVAKLEAK] will be synthesized on a normal non-dendritic peptide carrier backbone as well as to a double-attachment point substituted backbone by the methods outlined above. Fmoc-values will be determined and the Kaiser test will be performed after all coupling steps. The E_{280} of the final product will also be measured and compared to the theoretical value derived from the knowledge of the number of tyrosine residues in the molecule (see Perkins 1986). Finally, mass spectrometry and circular dichroism will be performed. The results are expected to show that the double-density coupling was possible by selecting the appropiate reaction conditions and that couplings progressively get harder to drive to completion as the length of the peptide

increases. Circular dichroism of the final, cleaved and dissolved product is expected to reveal an increased relative amount of α-helix compared to the normal derivatized non-dendritic peptide carrier.

Coupling of two different peptides to the same backbone.

1. By use of multiplied attachment points:

This can be accomplished by using lysine with an ε-amino protection group that is stable both to TFA and to piperidine. One such group is Dde ((1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-ethyl) which is cleaved by 2% hydrazine in NMP. Another such group is Aloc (allyloxy-carbonyl) which is cleaved by Pd(0)-catalyzed hydrogenation. These lysine derivatives are both commercially available. Coupling this amino acid to a non-dendritic backbone peptide and deprotecting the Fmoc-group with piperidine provides α-amino-groups for coupling of the first type of branch, which should be terminated with a Boc-α-aminoprotected amino acid. Then by 5 × 3 minutes treatment with 2% hydrazine hydrate in NMP, followed by NMP-wash, the ε-amino-functionality is available for coupling the other branch. The reverse order of operations is not possible, as Fmoc is not stable to hydrazine. Also, the HMB-linker is hydrazine-labile, but a short-time, low level exposure in an aprotic solvent like NMP probably does not affect the HMB-linker.

2. Inclusion of additional peptides in the backbone part of the non-dendritic peptide carrier or as side chains or parts of side chains:

Using the principles outlined above, different peptides or polypeptides may be included in a branched peptide complex using a non-dendritic backbone peptide, introduced by

-differently protected attachment points (two different peptides).
-linear combinations of different antigens in the branches.
-linear combinations of different antigens at the N- and/or C-terminals of the backbone-peptide.

If included in the backbone peptide, such additional peptides are adequately protected to avoid side-chain substitution in those parts of the backbone. For positioning at an orthogonal attachment point, the peptide should also be left in a protected state. If such a peptide is of universal use, it may be included in a general, pre-synthesized "branched backbone" structure. In one embodiment, the non-dendritic backbone peptide according to Example 1 was synthesized with a C-terminal extension consisting of the IL-1-β peptide VQGEESNDK, with a Dde-protected lysine (see Example 1, Table 1, structure nos. 4 and 5). This backbone peptide was then used to couple the antigenic HIV gp120 peptide, GEIKNCSF-NISTSIRGKVQKEYAFF (see Example 5) by direct synthesis and compared with the same construct without the IL-1-β peptide extension in the immunization experiment in examples below.

In the present example, using the non-dendritic backbone peptide disclosed in Example 1, a T-cell stimulatory peptide included in the list given in Example 4 will first be synthesised on the α-amino group, followed by palmitoylation and synthesis of an antigenic peptide on the ϵ-amino groups.

One such synthesis will be performed with the T-cell stimulatory peptide QYIKANSKFIGITE (Tetanus toxoid 830-843) on the α-amino group and GEIKNCSFNISTSIRGKVQKEYAFF (HIV gp120 peptide) as the antigenic peptide coupled to the ϵ-amino groups. In another synthesis, the same

antigenic peptide will be coupled in combination with the IL-1-β-derived stimulatory peptide VQGEESNDK.

Example 6

Indirect coupling: Attachment by coupling chemically presynthesized, blocked peptides.

Please note that while these examples show only syntheses employing single-attachment points, similar couplings are possible on multiple attachment points and differently protected attachment points as long as the introduction of additional, unblocked functional groups is avoided.

Coupling in the "N-terminal out" orientation

In an example synthesis, the peptide to be coupled will be synthesized, using Fmoc-chemistry on Novasyn KB, and cleaved from this solid-phase in its side-chain, and α-amino blocked form by aqueous base treatment leaving out piperidine- and TFA-treatments. This blocked peptide will be used directly without further purification, preactivated by TBTU/HOBT/NMM in NMP, and subsequently incubated in 10 times molar excess with the backbone peptide on the solid phase, until the Kaiser test is negative. To obtain complete coupling, rather than prolonging the coupling time for more than one hour, coupling will be repeated with a new portion of activated, blocked peptide.

Coupling in reverse orientation

In this example, the peptide to be coupled will be synthesized on the very acid-labile 3-(amino-4-methoxybenzyl)-4,6-dimethoxyphenyl-propionic acid linker coupled to a Novasyn base solid-phase, including a final piperidin-cleavage of the α-amino Fmoc-group, yielding the side-chain protected peptide as the amide.

This peptide will be coupled to the amino groups of a solid-phase bound standard non-dendritic backbone peptide carrier by glutaraldehyde or by EDC (see Example 7), washed, cleaved, and purified.

Example 7

Indirect coupling: Attachment by coupling chemically presynthesized or recombinantly expressed, side-chain non-blocked peptides

Here some examples on chemoselective ligation chemistries for unprotected peptides, being synthetic, or natural (proteolytic fragments, recombinant fragments, etc.), are presented. In the special case of synthetic or recombinant peptides, the freedom of being able to include specific amino acids, especially cysteine, at any desired position in the peptide chain greatly facilitates such couplings and expands the range of coupling strategies available. However, methods do exist to chemoselectively couple unprotected peptides of any sequence.

Coupling to standard backbone of unmodified peptide by temporary protection

Preferred temporary protection methods include citraconylation (primary amino group protection, the protecting group being released by low pH) and Fmoc-derivatization by Fmoc-succinimide (primary amino group protection, the protecting group being released by piperidine).

In one example, presynthesized peptides will be dissolved to 2 mg/ml in 0.1 M $NaHCO_3$, pH 9, and 10 µl pr ml citraconic anhydride (CA) will be added, and the pH readjusted to 9 with 1M NaOH, followed by another addition of CA. This will be repeated until the pH remained stable upon addition of CA. Incubation will then be performed overnight at 4^0C. Then 10 mg EDC (1-ethyl-3 [dimethyl (aminopropyl)] carbodiimide,

(from Pierce Ltd.)) will be added per mg peptide and incubated for 10 minutes before addition of a non-dendritic backbone peptide carrier, still attached to the solid phase. Subsequently, this suspension will be allowed to react under agitation for 2 hours at room temperature. The product will be worked up, using the procedure outlined in Example 1 (citraconic anhydride groups are removed by acid).

In another example, temporary protection will be achieved by Fmoc, introduced as the succinimide (1 equivalent) to a 1 M solution of the peptide in carbonate (1 equivalent) in water/acetone (1/1, vol/vol) incubating overnight under stirring. Then pH will be adjusted to 2 with conc. HCl and acetone will be removed *in vacuo*. The product will be taken up in chloroform and washed with 0.1 M HCl and recovered from the organic phase.

Coupling to standard backbone of Cys-substituted peptide by chemoselective ligation

Cysteine can be included at any position in a synthetic or recombinant peptide as long as it does not interfere with the reactivity of the peptide. It can, of course, also be introduced in the backbone peptide, preferably by attaching it to the ϵ-amino groups of the lysine residues. As shown in the examples below, this is important as the cysteine side-chain thiol group can be targeted selectively by a variety of chemical methods, leading to disulfide bonds or more stable thioether bonds, and even, in the special case of thiol reacting with thiocarboxyl, an amide bond is formed by rearrangement.

In a practical example, peptides to be coupled were synthesized with cysteine either at positions corresponding to cysteine positions in the natural sequence or in the part of the peptide that was preferred to be turned inward to the backbone peptide. The following peptides were coupled in this way to a non-dendritic peptide carrier, structure 1 (Table

1), or, in the case of IL-1, IFNγ-1, IFNγ-2, and TNFa, the lysine side-chain formerly protected with Dde in the structure 7 non-dendritic peptide carrier of Table 1:

VQGEESNDKC (IL-1β)
CVQGEESNDK (IL-1β)
CPSTHVLLTHTI (TNFα)
HGTVIESLESLNNYFNSSGIDVEEKSLFLDIWRNWQK(C) (IFNγ-1)
AKFEVNNPQVARAAFNELIRVVHQLLPESSLRKRKRSRC (IFNγ-2)
CGMTAEDLQTRYN (PalA)
CTEADYAKNRAVLEY (PalA)
CSGGKGSFDLEDV (Tbp-2)
CPKGGNYKYIGTWD (Tbp-2)
NGSVGAVFGAK (Tbp-2)
AELGGQFHHKSENG (Tbp-2)
CASQRDRFQVHNPHENDA (SIF)
CKSQSGIEKTTRILHHANISESTQQN (SIF)
CQATAKMAEEQLTTLHVRSEQQS (SIF)

After synthesis of these peptides on a modified Rink-resin followed by cleavage, analysis and purification, they were coupled to a non-dendritic backbone peptide in which the ε-amino groups were converted to thiols by reaction with SPDP in NMP at 20 times molar surplus (resulting in a negative Kaiser test). Peptides were coupled in 2-3 times molar surplus in NMP for 2 hours at room temperature. This coupling proceeded by the formation of disulfide bonds with a simultaneous release of pyridine-2-thione which can be measured spectrophotometrically at 343 nm to indicate the degree of substitution (molar extinction coefficient is 8080), and it was found that the substitution was above 85% of the theoretically available attachment points in the non-dendritic peptide carrier. Hereafter, the derivatized non-dendritic peptide carrier was liberated from the resin as usual.

In another synthesis, these and other cysteine containing peptides will be coupled to a bromoacetyl-modified backbone peptide as thioethers. Using the reverse approach, bromoacetylated peptides will also be coupled to a cysteine-derivatiz-

ed backbone peptide; in these peptides, the bromoacetyl group will be introduced by bromoacetic anhydride (on primary amines, typically on the α-amino group), or by using special amino acids (BBAL (Inman 1991)) with bromoacetylated side-chains during synthesis, to include the functionality at any site in the chain. With the bromoacetic anhydride method, natural peptide fragments will also be coupled, ensuring α-amino reactivity of the bromoacetic anhydride by keeping the pH at 6.0, performing the reaction in aqueous buffer at 0^0C, adding bromoacetic anhydride in organic solvent below 1/100 volume.

Coupling to standard backbone of Ser-substituted peptide by chemoselective ligation.

Specific N-terminal coupling of a Ser N-terminated (poly)-peptide can be obtained after oxidation under specific conditions. Such oxidation yields an N-terminal carbonyl function, specifically reactive with acylhydrazines (hydrazides) and with hydroxylamines, yielding hydrazones and oximes, respectively.

As an example, the peptide to be coupled will be synthesized with an N-terminal S, cleaved and purified, and oxidized in solution by 2 mM periodate in 50 mM imidazole/HCl, pH 6.9 for 10 minutes before SepPack purification. Then the peptide will be reacted with a hydrazide-derivatized non-dendritic backbone peptide carrier (in which free amino groups have been derivatized to hydrazides by TBTU/HOBt/NMM-mediated coupling of Boc-monohydrazide succinic acid) in solution overnight in an aqueous buffer at a pH between 4 and 5, using two hydrazide equivalents for each oxidised peptide to be coupled. Reduction of the formed hydrazone to the substituted hydrazine will be performed for two days with 0.2 M cyanoborohydride at pH between 4 and 5.

Example 8

Indirect coupling: Attachment by coupling presynthesized, side-chain non-blocked peptides by C-terminal-selective chemistry

By way of chemoselective coupling, it is possible to introduce presynthesized peptides on a non-dendritic peptide carrier (NDPC) without needing to employ side-chain or α-amino protection. In this example, the NDPC has to be derivatized in order to obtain the desired selective reactivity.

In a planned first example, the peptide to be coupled will be synthesized on a Novasyn KB-resin and then cleaved after the final piperidine treatment followed by a TFA-treatment. Cleavage will be performed by hydrazine in dioxane/methanol as detailed in Example 4, to yield the peptide hydrazide, which will then be purified by preparative reverse phase HPLC. The NDPC will be derivatized with S (serine) on all attachment points, periodate oxidized to yield α-oxoacyl-groups (neutral pH in imidazole buffer, see Example 7) and then reacted with the peptide-hydrazide at a pH between 4.5 and 5, followed by cyanoborohydride reduction of the hydrazone to hydrazine as shown in Example 7 (all operations performed on the solid phase attached peptide).

In another possible example, introduction of carbonyl groups on the NDPC may be accomplished with 2,2-dimethoxyacetic acid, being deprotected with concentrated HCl.

In yet another planned example, the peptide to be coupled may be synthesized as its thiocarboxylic acid on a solid phase, using the special linker of Yamashiro (Yamashiro 1988, used by Schnölzer 1992, and Dawson 1994), 4-[(Boc-aminoacyl)thiobenzyl]-phenoxy acetic acid, yielding the thiocarboxylic acid upon cleavage with HF/10% p-cresol. After purification, the peptide thiocarboxylic acid is coupled to a solid-phase bound NDPC that is further derivatized with bromoacetic acid to

yield bromoacetyl-substituted amino groups as attachment points. The final product is expected to contain the branch-peptides attached as stable thioesters.

Example 9

Attachment of naturally-derived peptides

Chemical unambiguity is most easily obtained by a high degree of chemical selectivity. This way a defined orientation and stoechiometry is obtained. This is most easily obtained if the natural peptide has a cysteine or a N-terminal serine that can be used for coupling by the methods given above. If this is not the case, peptides may be temporarily protected and coupled as shown in Example 7. If this is not suitable, some selectivity can be obtained, using conventional coupling methods, that is e.g. glutaric aldehyde (coupling to amino- and thiol groups), carbodiimides (coupling to amino groups), and m-maleimide benzoyl-N-hydroxysuccinimide esters (coupling from amino- to thiol-groups), by optimizing relative reactant concentrations, pH, temperature and solvent. This may also be combined with a purification step. Another example is the use of haloacetic anhydride to selectively introduce e.g. bromo- or chloroacetyl groups at the α-amino group only in a free peptide. This group will then react with thiol groups.

In an example, this will be performed with a presynthesized peptide as a model of a naturally occurring peptide. The peptide will be bromoacetylated in solution in 0.1 M 2-(*N*-morpholino)ethane sulfonic acid, pH 6.0, using bromoacetic anhydride prepared from the acid by DCC-mediated activation, in NMP, added at 1/100 (vol/vol) to the aqueous peptide solution, and incubating for 3 x 3 minutes at room temperature (adding new reagent each time). After purification, the peptide will be coupled to a cysteine-derivatized standard backbone peptide until negative Ellman-test.

Example 10

Attachment of carbohydrates by coupling naturally-derived carbohydrates

In these experiments, a carbohydrate immunogen is derived from *Salmonella typhimurium LPS* (lipopolysaccharide). The salmonella LPS is cleaved by mild acid treatment into its lipid part (insoluble precipitate) and its carbohydrate part (soluble). The soluble LPS carbohydrate is obtained after centrifugation and then oxidized by sodium metaperiodate for 10 min. at 0.1 M in the dark, followed by rapid desalting on a Pharmacia PD-10 column. Also N-acetyl-D-galactosamine is coupled. Both the LPS oligosaccharide and N-acetyl-D-galactosamine constitute well-known carbohydrate epitopes and may be shown by monoclonal antibodies to remain intact after coupling as decribed below.

Coupling to non-dendritic peptide carrier

Blomberg's methodology (Blomberg 1993) is used to introduce the carbohydrate at amino groups in a non-dendritic backbone peptide. The oxidized carbohydrate is allowed to react in molar excess with the solid-phase bound backbone peptide in DMSO overnight at $60^{0}C$, followed by cooling to room temperature and reaction with acetic anhydride (10 equivalents) for 4 hours at room temperature.

In another embodiment, N-acetyl-D-galactosamine is coupled by its reducing end to the non-dendritic peptide carrier (NDPC) to constitute a Tn-antigen that is reactive with a monoclonal antibody against Tn-antigen.

The retention of the antigenic structure of the carbohydrate is shown by performing an ELISA, coating with the easily coatable carbohydrate derivatized NDPC, and detecting it with a mouse monoclonal antibody against *Salmonella typhimurium* LPS carbohydrate (O-chain specific antibodies) in the first

embodiment and with the monoclonal antibody directed against Tn-antigen in the second embodiment.

Coupling to hydrazide-terminated "anchoring polymer"

In a possible example, the oxidized carbohydrate is mixed at room temperature with the backbone peptide hydrazide (see Example 4) at 1/1 mol/mol in 0.1 sodium acetate, pH 5.5, and incubated for 1 hour. The resulting conjugate is easily purifiable by Sephadex G50 gelfiltration, monitored at 280 nm. HPLC-analysis will show that it elutes before the nonconjugated peptide on a C-18 reverse phase column (running conditions as decribed for Fig. 1). The retention of the antigenic structure of the carbohydrate is shown by performing an ELISA, coating with the easily coatable lipopeptide-carbohydrate complex, and detecting it with a mouse monoclonal antibody against *Salmonella typhimurium* LPS carbohydrate (O-chain specific antibodies).

In another example, N-acetyl-D-galactosamine is coupled by its reducing end to the NDPC to constitute a Tn-antigen, that is reactive with a monoclonal antibody against Tn-antigen.

Another planned example involves the use of the following chemical strategy for attaching the same carbohydrate antigens:

The carbohydrate is derivatized with a general amino- and hydroxyl-reactive substance such as a carbodiimide, divinylsulfone or cyanogen bromide. This is followed by reaction with a diamine spacer such as, e.g., 1,4-butanediamine, and, subsequently, the derivative is bromoacetylated and then reacted with a cysteine-derivatized NDPC. In another embodiment, the said derivative is reacted with SPDP before reaction with the cysteine-derivatized NDPC.

Coupling to hydrazide-substituted standard-backbone

In a possible example, this is done as above, using a non-dendritic carrier peptide derivatized with Boc-monohydrazide succinic acid to carry hydrazide groups as attachment points.

Example 11

Attachment of a hapten

Haptens are useful as labels for model experiments as they can be detected specifically either by commercially obtainable antibodies (with the haptens digoxigenin and trinitrophenyl) or by avidin or streptavidin (with the hapten biotin). Biotin was easily incorporated into the non-dendritic peptide backbone either using the succinimide ester or activating free biotin by TBTU/HOBt/NMM in NMP before coupling to the amino groups. Digoxigenin could also be coupled as the succinimide. Trinitrophenylsulphonic acid reacted rapidly with amino groups to introduce triphenyl groups. In all cases, the reaction could be followed by the Kaiser test.

Example 12

Attachment of DNA/RNA by coupling

DNA or RNA may be incorporated into the non-dendritic peptide carrier or coupled as any other antigenic moiety as a branch, part of a branch, or as an "orthogonal" branch, condensing the aldehyde of a C-formyl nucleoside to form an imine linkage which is reduced by reductive alkylation to form a methyl alkylated amine bond (Vasseur 1992). Subsequently, the resulting 3'-bound nucloside can be prolonged to an oligonuclotide using automated DNA-synthesis, preferably coupling the DNA before peptide and using the coupled DNA either directly for encoding an interesting peptide or for binding a piece of "natural" DNA to be expressed in the host by hybridization to the non-dendritic peptide carrier or for direct stimulation as with the CG oligonucleotide of Klinman (1996). In a typi-

cal application, only one DNA-copy is coupled per non-dendritic peptide carrier (NDPC).

In one further possible example, an oligonucleotide corresponding to a T-cell stimulatory peptide will be coupled to one specific lysine selectively deprotected side-chain using Dde or Mtt as an orthogonal protecting group to Boc. Subsequently, the rest of the lysine side-chains is deprotected by TFA-treatment and antigenic peptides are coupled directly by the methods outlined above. Antigenic peptides to be used may include GEIKNCSFNISTSIRGKVQKEYAFF (gp120 peptide), and the DNA-encoded T-cell stimulatory peptide may be, e.g., QYIKANSKFIGITE (tetanus toxoid peptide). In a further synthesis, a piece of binding DNA will be incorporated into the NDPC to bind the polyepitopic DNA defined by Thomson (1995) as mentioned in Example 4.

Example 13

Attachment of DNA/RNA by non-covalent inclusion

In this proposed example, a non-derivatized peptide carrier (NDPC) will be derivatized at one specific Dde-protected lysine side-chain with a polylysine chain consisting of 10 and 15 Dde-protected lysines (two different experiments), the N-terminal lysine being capped by acetic anhydride. This chain will be left in the protected state, while the rest of the backbone lysine side-chains is deprotected and used for coupling of an antigenic peptide including GEIKNCSFNISTSIRGKVQKEYAFF (gp120 peptide). Hereafter, all side-chain protecting groups will be cleaved, and the peptide complex will be released, purified, and mixed in an aqueous buffer at neutral pH and with a moderate ionic strength with the appropiate DNA in a purified state or in a defined mixture with other defined DNAs. One such DNA corresponds to the tetanus toxoid T-cell peptide QYIKANSKFIGITE. Another such mixture is a mixture of DNAs each corresponding to a promiscous T-cell

epitope. Yet another DNA to be employed is the "poly-epitope" DNA of Thomson (1995) mentioned in Example 4.

Example 14

Attachment of DNA/RNA by hybridization to PNA (peptide nucleic acid)

These planned syntheses will proceed as in Example 12, using peptide nucleic acids (PNA, WO 95/01369) as building blocks on the selectively deprotected lysine side-chain defining the PNA sequence to be able to bind by hybridization the tetanus toxoid DNA corresponding to the T-cell stimulating peptide QYIKANSKFIGITE, and, in another experiment, the polyepitope DNA of Thomson (1995) mentioned in Example 4.

Example 15

Attachment of DNA/RNA by binding to peptide-bound intercalators

Another way to bind nucleic acids is to enclose in the structure an intercalator. This will be done by coupling quinoline as a thioether to a cysteine side-chain (Brown 1994) included in the C-terminal part of the non-dendritic peptide carrier. The cysteine thiol will be deprotected before Fmoc-deprotection to allow for substitution with quinoline. Hereafter, synthesis will be continued and, as the last step, lysine side-chains will be deprotected and used for coupling of an antigenic peptide including GEIKNCSFNISTSIRGKVQKEYAFF (gp120 peptide). Hereafter, all side-chain protecting groups will be cleaved, and the peptide complex will be released, purified, and mixed in an aqueous buffer at neutral pH and with a moderate ionic strength with the appropriate DNA in a purified state or in a defined mixture with other defined DNAs. One such appropriate DNA is the DNA corresponding to the tetanus toxoid T-cell peptide QYIKANSKFIGITE. Another such mixture is a mixture of DNAs each corresponding to a

promiscous T-cell epitope. Yet another DNA is the "poly-epitope" DNA of Thomson (1995) mentioned in Example 4.

Example 15A

The use of a non-dendritic peptide carrier for coupling of antigens illustrating its use in a kit

The use of the non-dendritic peptide carrier (NDPC) in a kit format is illustrated by the following planned example:

An NDPC will be synthesized on a RINK-MBHA-type solid phase from Novabiochem to a substitution of from about 0.05 μmoles/g to 0.2 μmoles/g, preferably between about 0.05 to 0.1 μmoles/g. The NDPC will be included in the sequences listed in Example 1 and will, for illustrative purposes, include a normal NDPC without any auxiliary segments in addition to NDPCs containing one side-chain protected auxiliary segment selected from a tuftsin tetramer, the interleukin-1 nonapeptide (VQGEESNDK), and T-cell stimulatory peptides mentioned in Example 4. After synthesis, attachment points will be created by cleavage of side-chain protection groups that are protected orthogonally to the other side-chain functionalities of the NDPC (see Example 1).

In one example, the NDPC-solid-phase will then be derivatized with SPDP, washed, dried, and stored for different time periods until tested for the ability to bind Cys-containing peptides as measured by the release of pyridin-2-thion and by the ability to react with antibodies against the immobilized peptide. Peptides to be immobilized will include the Tbp-2--peptide of Example 5 and a biotinylated peptide which can be detected by avidin.

Also, an NDPC derivatized with bromoacetyl groups will be used for the attachment of the same Cys-containing peptides.

In other examples, peptides will be attached by EDC, DSS, and by carboxylic activation with TBTU/HOBt/NMM or other equivalent activation procedures. Also, blocked peptides will be attached.

In addition, the attachment of haptens, including biotin and digoxigenin, and carbohydrates will be shown.

In all cases, the final complex will be liberated by TFA and analysed by HPLC.

Such conjugations are expected to be performed in a quick two-step procedure consisting in a conjugation reaction followed by liberation and work up as usual.

Example 16

Preparing Immune-stimulating Complexes (Iscoms) containing derivatized non-dendritic peptide carriers

Iscoms are easily prepared using standard methods (Morein 1984, Mowat 1992, Current Protocols in Immunology, 1992, section IV: "Preparation of Immune Stimulating Complexes", 2.11.1-2.11.12), and lipopeptides insert themselves with a minimal perturbation of the Iscom structure and orientating their hydrophilic parts outwards.

A non-dendritic backbone peptide carrier will be synthesized as above, side-chain deprotected, and used for coupling or synthesizing side-chain antigenic peptides before cleavage. After purification, peptides will be incorporated into Iscoms by mixing with cholesterol, phosphatidylcholine, and quil A, according to standard procedures referred to above.

As an illustrative example, the non-dendritic peptide carrier palmitate-GKGKGKGKGKGG was synthesized in a 130 μmol/g scale on a Pepsyn KB solid phase (on the base-labile 4-hydroxymethyl benzoic acid linker). After completion of synthesis,

Boc-protection groups on the K-residues were removed by a 2 × 15 minutes wash in 95% TFA/water with a concurrent change from negative to a positive Kaiser test. After neutralization and wash in NMP, biotin was attached by TBTU/HOBt/NMM, using a 2 times surplus of biotin and 2 couplings of 30 minutes at room temperature preceeded by 10 minutes of preactivation. After washing in NMP and water, the side-chain biotinylated peptide was cleaved by NaOH/water as in Example 1, worked up by desalting on preparative HPLC followed by freeze-drying, and then used without further purification for inclusion into Iscoms by mixing the peptide with the Iscom-forming reagents (peptide/quil A = 1/5, w/w) following standard procedures (see above). Iscoms were characterized by sucrose-gradient ultracentrifugation, and fractions were analysed by electron microscopy for Iscom-formation and by HRP-streptavidin ELISA for biotin. The Iscom-containing fractions showing well-defined normal Iscom-structures by electron microscopy were found by the ELISA to contain also the biotin-peptide in a presentation where the biotin was accessible to the reaction with the HRP-streptavidin-reagent. The purified Iscom-fraction retained avidin-reactivity even after extensive dialysis, which was easily shown by dot-blotting. Furthermore, peptides were demonstrated in the purified Iscoms by direct reverse-phase HPLC analysis of the Iscoms.

Example 17

Preparing and using loaded Iscoms for attachment of antigenic moieties.

Carrier with amino groups

In one embodiment, the non-dendritic peptide carrier (NDPC) lipopeptide of the invention is included in an Iscom before coupling antigenic branch-moieties. This allows the preparation of ready-made "loaded" Iscoms, ready for en-bloc coupling of branch-moieties in a manner which is special by

employing a synthetic and specifically designed NDPC lipopeptide as the Iscom-inserted carrier.

A standard backbone-lipopeptide will be synthesized by the methods given above, deprotected, cleaved from the solid phase, purified, lyophilized and solubilized, and incubated together with Iscom-forming substances according to Morein (1984). In a series of incubations, the peptide-to-quil A ratio will be varied around 1:5, such as from 1:2 to 1:10. After preparation of Iscoms, they will be analysed for integrity and general appearance by electron microscopy, for accessibility of backbone-peptide amino groups by hapten-derivatization of free amino groups (using gold-labelled immunoglobulin indirectly binding to trinitrophenyl groups introduced on the amino groups, or by gold-labelled avidin labelling biotinylated amino groups) followed by inspection by electron microscopy and for loading "density" (amount of peptide pr. quil A) by quantitative HPLC of peptide-loaded Iscoms.

In one particular embodiment, the NDPC will be synthesized on a solid phase using a water-cleavable linker (see Hoffmann, 1994). Then the peptide is expected to be obtained in its Iscom-enclosed form simply by mixing the finished, but still solid-phase bound peptide with Iscom-forming agents in aqueous buffer for between 2-24 hours, then filtering off the solid-phase.

Compatible coupling schemes for the subsequent coupling of antigenic branch structure include methods that do not involve the use of organic solvents and/or extremes of pH (e.g. Regenmortel 1988). As examples, peptides included in the list in Example 5 will be coupled using such coupling schemes. The resulting peptide-loaded Iscoms will be analysed by electron microscopy and by HPLC for loading "density".

Carrier with a C-terminal hydrazide for coupling oligosaccharides

A backbone-peptide containing a C-terminal hydrazide, preferably obtained by cleaving the peptide from the solid-phase by hydrazine/NMP as detailed above (Novabiochem 1994), will be included as such in an Iscom, simply by mixing the components in PBS and incubating at room temperature for 24 hours, then purifying Iscoms by sucrose gradient-centrifugation. The carbohydrate moiety will be either N-acetyl galactosamine or natural or oxidized Salmonella LPS O-carbohydrate (see Example 10) and will be attached as detailed in Example 10. Iscom will be inspected by electron microscopy and further analysed by studying the binding to the Iscoms of a number of carbohydrate-specific antibodies, including anti-Tn antibodies and anti-LPS antibodies both by gold-labelled antibodies in electron microscopy and by ELISA using the loaded Iscoms for coating.

Example 18

Application of a non-dendritic peptide carrier for immunization to create protection against virus: The mink enteritis virus model

In this model, the virus capsid peptide, DGAVQPDGGQPAVRNER, originally derived from canine parvovirus constitutes a protective epitope (Langeveld 1995) against mink enteritis virus induced disease.

In the example, this peptide will be coupled in the "C-outward" orientation by including a N-terminal cysteine and coupling to a SPDP-derivatized non-dendritic peptide carrier. "N-outward" orientation will be achieved by normal sequential synthesis on the same type of backbone peptide.

As the non-dendritic backbone peptide carrier, the backbone peptide no. 6 of Table 1 will be used as well as the same peptide extended C-terminally with either

-a tetanus toxoid T-cell stimulatory peptide, TT (QYIKANSKFI-GITE),
-the IL-1ß nonapeptide (VQGEESNDK), or

-a tuftsin tetramer ($[TKPR]_4$)
using Mtt-protected side-chains in attachment point-lysine residues and Boc-protection for other lysine residues.

After synthesis of peptides corresponding to these four groups and HPLC and MS analyses, immunization will be performed by one subcutaneous injection of 50-200 µg peptide-construct in 1000 µl PBS in the following groups of animals:

Backbone $[C\text{-outward}]_4$
Backbone $[N\text{-outward}]_4$
Backbone $[C\text{-outward}]_4$ + Alhydrogel
Backbone $[N\text{-outward}]_4$ + Alhydrogel
Backbone-TT peptide $[C\text{-outward}]_4$
Backbone-TT peptide $[N\text{-outward}]_4$
Backbone-tuftsin $[C\text{-outward}]_4$
Backbone-tuftsin $[N\text{-outward}]_4$
Backbone-IL-1 $[C\text{-outward}]_4$
Backbone-IL-1 $[N\text{-outward}]_4$
(10 groups of animals, 4 in each group)

Immunizations will be followed by whole virus challenge at day 21, and animals will be checked for infection (isolation of virus) and clinical disease for 14 days after challenge. Titers will be followed throughout by ELISA employing the relevant immunization peptide as the coating antigen.

Example 19

Non-dendritic peptide carriers for use as diagnostics in the diagnosing of infectious diseases

The use of the non-dendritic peptide carrier in a diagnostic immunoassay is illustrated by the following example in which peptide sequences derived from an infectious agent are being recognized by antibodies present in samples from humans exposed to the infectious agent.

Diagnostic immunoassay for detection of HIV-1 and HIV-2 infection

Test-serum panels

Test serum panels from Boston Biomedica, Inc. are used throughout the diagnostic testing.

Preparation of non-dendritic peptide carriers carrying peptides specific for HIV-1 and HIV-2

The following peptide sequence specific for HIV-1 and often recognized by HIV-1 infected patients is selected for HIV-1:

gp41 (aa598-609):

Leu-Gly-Ile-Trp-Gly-Cys-Ser-Gly-Lys-Leu-Ile-Cys

The following peptide sequence specific for HIV-2 and often recognized by HIV-2 infected patients is selected for HIV-2:

gp36 (aa587-605):

Leu-Gln-Asp-Gln-Ala-Arg-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys-His-Thr

Different diagnostic components are produced by coupling peptide sequences to non-dendritic peptide carriers following the procedure described in Examples 1 and 2.

The non-dendritic peptide carrier provides a multimer presentation of each of the peptide sequences which can then be used in a variety of diagnostic assays, including ELISA, line-blotting, and agglutination assays.

Each non-dendritic peptide carrier carrying the HIV specific peptides is tested in parallel with the same peptide not linked to the carrier peptide and with the corresponding recombinant protein HIV-1 gp41.

ELISA-assay

Synthetic peptides were coated on Maxisorp microtitre plates (Nunc, Roskilde, Denmark). Peptides (10 µg/ml) were coated to the plates in 100 mM $NaHCO_3$ at pH 9.6 or in 0.1 mM glycin-HCl at pH 2.5. All coatings were performed overnight at 4°C. The wells were washed 4 times in 0.5 M NaCl, 3mM KCl, 1mM KH_2PO_4, 8mM $Na_2HPO_4,2H_2O$, 1% Triton X-100. This washing procedure was repeated after each of the following incubation steps:

1) Plasma samples, 1% (v/v) in incubation buffer (washing buffer plus 15 mM bovine albumin, pH 7.2), were incubated for 1 hour at room temperature.

2) 100 µl per well of horse radish peroxidase-conjugated rabbit anti-human class-specific antibodies (DAKO, Copenhagen, Denmark) diluted in incubation buffer, was added at room temperature for 1 hour.

Enzyme activities were quantitated after addition of 100 µl per well of 0.67 mg/ml 1,2-phenyldiamine hydrochloride (DAKO) dissolved in 100 mM citric acid-phosphate buffer, pH 5.0 containing 0.015% (v/v) H_20_2. The reactions were stopped by

adding 50 µl per well of 2.5 M H_2SO_4, and the optical densities were measured in an ELISA scanner at 492 nm.

All tests were done in duplicate.

Result

The ELISA procedure was optimized by testing different concentrations of the peptides alone and coupled to the carriers, respectively. Fig. 5 shows antibody reactivity of a HIV-1 seropositive donor against dilution curves of the gp41 (aa598-609) peptide, being tested alone and linked to the carrier. The ELISA procedure is optimized by testing different concentrations of the peptides.

Fig. 6 shows dilution curves of sera obtained from HIV-2 seropositive donors and HIV-2 seronegative donors tested against the non-dendritic peptide carrier derivatized with gp36 (aa 587-605) peptide. Positive signals were obtained at all dilutions of HIV-2 seropositive sera tested while the seronegative control sera were negative in all the dilutions tested.

Fig. 7 shows the results with fixed dilutions of sera obtained from a panel of HIV-2 and HIV-2 seronegative donors tested against gp36 peptide alone and coupled to the non-dendritic peptide carrier. The sera were also tested against recombinant protein HIV-2 gp36. All the seropositive donor sera were reactive in the assay using gp36 peptide coupled to the non-dendritic peptide carrier, while no reactivity was detectable with the seronegative control sera.

Fig. 8 shows dilution curves of sera obtained from one HIV-2 seropositive donor and one HIV-2 seronegative donor tested against the gp36 peptide alone and coupled to the non-dendritic peptide carrier. The sera were also tested against recombinant HIV-2 gp36. The peptide coupled to the carrier

was recognized at higher dilutions of the seropositive serum compared to the peptide alone.

The HIV-2 specific gp36 peptide coupled to the non-dendritic peptide carrier without any modifications was as useful for diagnostic purposes as was the whole recombinant HIV-2 gp36 protein. The gp36 peptide coupled to the non-dendritic carrier was recognized by HIV-2 seropositive sera at lower dilutions than the peptide alone.

Dot-blotting assays for diagnosing for HIV-1 and HIV-2:

Procedure

The non-dendritic peptide carrier derivatized with HIV peptides (app. 50 µg/ml) as well as the peptide alone is coated on nitrocellulose membranes in 2.5 µl volumes. Blocking of nitrocellulose is performed for 10 min with 0.05 M Tris buffer, pH 7.4 + 0.5 M NaCl + 0.5% Tween 20. Test-sera are incubated for one hour diluted in assay-buffer: Tris buffer, pH 7.4 + 0.5 M NaCl + 0.05% Tween 20. After washing, the nitrocellulose membrane is incubated two times with assay-buffer, incubation with peroxidase-conjugated anti-human immunoglobulin follows. Human immunoglobulin reactivities with peptides are detected using the substrate 3-amino-9-ethylcarbazole. The test results are inspected visually.

1st Screening

First screening is performed with a panel of sera from app. 10 HIV-1 patients, 5 HIV-2 patients, and 50 uninfected individuals.

Sensitivity and specificity of the assay are estimated, and the reproducibility of the assay is tested by repeated tests of the serum samples.

2nd Screening

Second screening is performed using 14 sera with low titers of antibodies against HIV-1 virus. This test using sera with low or uncertain reactivities against HIV allows for evaluation of the sensitivity and specificity of the non-dendritic peptides in detecting antibodies against HIV infection as compared to that of other commercial assays.

3rd Screening

Third screening is performed using sera from app. 5 donors from which several blood samples have been collected around the time of sero conversion following infection. This test estimates the size of time-window from exposure to sero conversion at which point people start producing HIV-1-specific antibodies

Example 20

Non-dendritic peptide carriers derivatized with immunogenic peptides for use as vaccines

The non-dendritic peptide carrier derivatized with immunogenic peptides can be used for immunization purposes to stimulate antibody and T-cell responses against any immunogenic agent. The non-dendritic peptide carrier can thus be used for vaccination purposes to protect against pathogenic microorganisms. One example of this use of the non-dendritic peptide carrier is given below where mice are immunized against the parasitic disease malaria.

Immunization of mice with a non-dendritic peptide carrier derivatized with a peptide sequence from the erythrocyte binding antigen-175 from *Plasmodium falciparum*

Materials and methods

Mice were immunized with a non-dendritic peptide carrier derivatized with the synthetic peptide:

TLTKEYEDIVLKSHMNRESDD

This peptide is derived from the malaria parasite *Plasmodium falciparum*. The peptide covers a sequence of the erythrocyte binding antigen-175 (EBA-175) involved in the parasite invasion of erythrocytes. However, the sequence is normally not recognized by the immune system during infections.

Experimental design

Female 6-8 weeks old (C57BlxBALB/c)F1 mice were used in these studies.

BCG primed mice were immunized intraperitoneally 3 times, day 0, 21, and 49, with 16 µg of the non-dendritic peptide carrier derivatized with EBA-175 peptide conjugated to Purified Protein Derivative (PPD) with and without absorption to aluminium hydroxide.

In the second set of experiments, mice were immunized with the non-dendritic peptide carrier derivatized with the EBA-175 peptide without any conjugations. Some mice were immunized intraperitoneally 3 times, day 0, 21, and 49, with 16 µg of the derivatized non-dendritic peptide carrier, while some other mice were immunized subcutaneously 3 times, day 0, 21, and 49, with 16 µg of the derivatized non-dendritic peptide carrier mixed 1+1 with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant.

The mice were bled on days -1, 12, 33, and 61. Sera were collected from the bleedings and tested in ELISA for antibody reactivity against the peptide used for immunization.

Serum antibody reactivity with synthetic peptides in ELISA

The synthetic peptide conjugated to ovalbumin (1 µg/ml) was coated in 100 mM NaHCO-3, pH 9.6 on Maxisorp microtitre plates (Nunc, Roskilde, Denmark). All coatings were performed overnight at 4°C. The wells were washed 4 times in 0.5 M NaCl, 3mM KCl, 1mM KH_2PO_4, 8mM Na_2HPO_4, 1% Triton X-100. This washing procedure was repeated after each of the following incubation steps:

1) Mice sera made to 1% (v/v) in incubation buffer (washing buffer plus 15 mM bovine albumin, pH 7.2) were incubated 1 hour at room temperature,

2) 100 µl per well of biotinylated rabbit anti-mice IgG antibodies (Amersham) diluted in incubation buffer, and

3) 100 µl per well of streptavidin peroxidase (DAKO, Copenhagen, Denmark) diluted in incubation buffer was added at room temperature for 1 hour.

Enzyme activities were quantitated after addition of 100 µl per well of 0.67 mg/ml 1,2-phenyldiamine hydrochloride (DAKO) dissolved in 100 mM citric acid-phosphate buffer, pH 5.0 containing 0.015% (v/v) H_20_2. The reactions were stopped by adding 50 µl per well of 2.5 M H_2SO_4, and the optical densities were measured in an ELISA scanner at 492 nm.

On each ELISA microtitre plate, one positive control serum and one negative control serum were assayed as well as control wells without serum (background). All tests were done in duplicate.

Results

Fig. 9 shows that mice produced antibodies in response to immunization with the non-dendritic peptide carrier derivatized with EBA-175 peptide-PPD conjugate. The strongest anti-

body response was detectable after 3 immunizations. Absorption to aluminium hydroxide enhanced antibody production after one immunization but not after two or three immunizations.

Fig. 10 shows that mice produced peptide-specific antibodies in response to subcutaneous immunization with the non-dendritic peptide carrier derivatized with EBA-175 peptide mixed with Freund's adjuvant. The response was detectable after two immunizations. Some mice showed a weak peptide-specific antibody reactivity in response to the non-dendritic branched peptide alone after 3 immunizations intraperitoneally.

IgG1 reactivity is a marker of Th2 reactivity, while IgG2a reactivity is a marker of Th1 reactivity.

Mice immunized with the non-dendritic peptide carrier derivatized with EBA-175 peptide and Freund's adjuvant showed both IgG1 and IgG2a reactivity to the peptide (Fig. 14 and 15).

Immunization with two different non-dendritic peptide carriers each derivatized with a peptide sequence from HIV-1

Mice were immunized with a non-dendritic peptide carrier derivatized with the peptide aa 152-176 from HIV-1 gp120 having the amino acid sequence:

GEIKNCSFNISTSIRGKVQKEYAFF

This peptide has known B- and T-cell epitopes.

Another peptide for use as a vaccine linked to the non-dendritic peptide carrier is the peptide from HIV-1 gp41 comprising the following amino acid sequence:

LERLLL

This peptide has interleukin-2 homology (Reiher III, 1986).

Experimental design

Female 6-8 weeks old mice, BALB/cJ, were used in these studies.

One group of mice was immunized intraperitoneally 3 times, day 0, 21, and 49, with 16 μg of the non-dendritic peptide carrier derivatized with the HIV-1 peptides, while another group of mice was immunized subcutaneously 3 times, day 0, 21, and 49, with 16 μg of the non-dendritic peptide carrier derivatized with the HIV-1 peptides mixed 1+1 with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant.

The mice were bled on days -1, 12, 33, and 61. Sera were collected from the bleedings and tested by ELISA for antibody reactivity against the peptide, using ELISA assays as described for the malaria peptide.

Results

Fig. 11 shows that mice responded to the gp120 peptide after subcutaneous immunization with the derivatized non-dendritic peptide carrier mixed with Freund's adjuvant. The strongest antibody response was detectable after 3 immunizations.

The mice responded weakly to the gp41 peptide after intraperitoneal immunization with the non-dendritic peptide carrier derivatized with the gp41 peptide alone (Fig. 16). Some mice received two non-dendritic peptide carriers derivatized with gp-120 and gp-41 peptides in combination. These mice produced GP-41 specific antibodies (Fig. 16).

Mice also showed reactivity to the recombinant proteins gp120 and gp41 in response to subcutaneous immunization with the non-dendritic peptide carrier derivatized with gp-120 peptide

mixed with Freund's adjuvant respectively two non-dendritic peptide carriers derivatized with gp-120 peptide and gp-41 peptide mixed with Freund's adjuvant (Fig. 17 and 18).

Mice produced gp-120 peptide-specific IgG1 and IgG2a antibodies in response to subcutaneous immunization with the non-dendritic peptide carrier derivatized with gp120 peptide mixed with Freund's adjuvant, but the IgG2a responses were eliminated if the non-dendritic peptide carrier derivatized with gp120 peptide was mixed with the non-dendritic peptide carrier derivatized with gp41 peptide during the immunizations (Fig. 19 and 20).

Mice produced gp-41 peptide-specific IgG1 and IgG2a antibodies in response to subcutaneous immunization with a mixture of two non-dendritic peptide carriers derivatized with the gp-41 peptide and the gp-120 peptide mixed with Freund's adjuvant, while no response was detectable after subcutaneous immunization with the non-dendritic peptide carrier derivatized with the gp-41 peptide mixed with Freund's adjuvant alone (Fig. 21 and 22). The gp-120 peptide functioned as a carrier for the gp41 peptide providing T-cell stimulation to induce IgG reactivity to the gp41 peptide.

Other vaccine constructions

Repetition of the amino acid sequences

To evaluate the importance of repeating the amino acid sequence in the immunogenic peptide, the peptides $(VTEEI)_1$, $(VTEEI)_2$ and $(VTEEI)_3$ will be tested following the same procedure as described for the other malaria peptides.

Inclusion of B-cell and T-cell epitopes

The immunogenicity of the peptide construct may be further improved in several ways such as by the inclusion of one or more B-cell and T-cell epitopes as peptides in the non-

dendritic peptide carriers in addition to the molecule against which immunity is desired.

Inclusion of universal T-cell epitopes may have the effect of overcoming MHC restriction. This aspect will be tested by linking peptides from T-cell epitopes to the non-dendritic peptide carrier in combination with a peptide against which immunization is desired. Combinations of immunogenic peptides and peptides having T-cell epitope function will be used both based on the use of the peptides:

QYIKANSKFIGITEL

and

FNNFTVSFWLHRVKVSASHLE

Both of these sequences are known to be universal T-cell epitopes (see Wang, 1995) and will be used in combination with:

$(QGPGAP)_4$ (malaria specific)

and with the TCTKEYEDIVLKSHMNRESDD

(from the erythrocyte binding antigen-175).

Testing of the peptides will be performed using the experimental design described above under Example 20.

Other interesting epitopes

Likewise, a number of other interesting epitopes may be included in the non-dendritic peptide carrier alone or in combinations. Such interesting epitopes include Tcyt epitopes for stimulation of cytotoxic T-cells, lipids or tuftsin sequence for activation of the immune system, and cytokines or bioactive cytokine sequences.

Recombinant or native immunomodulators such as cytokines may be inserted into Iscoms together with non-dendritic peptide carrier constructs. The cytokines may include e.g. interleukin 1-18, interferon-gamma, interferon-alpha, interferon-beta, and TNF (tumour necrosis factor). Alternatively, bioactive cytokine-specific amino acid sequences may be part of the sequence included in the branched peptide-construct.

A number of other HIV-1 peptides can also be used following the experiment described above. Such peptides include various other peptide sequences from gp120 and gp41.

Example 21

Non-dendritic peptide carriers for use as therapeutic agent in therapy of various diseases

Non-dendritic peptide carriers derivatized with peptide sequences capable of preventing or curing diseases or diminishing disease manifestations constitute a very interesting aspect of the invention.

To illustrate this application of the invention, an example is given below:

Non-dendritic peptide carriers for treatment of sepsis-like disease

Sepsis-like disease manifestations are induced by phospholipids from microorganisms like lipid A of gram-negative bacteria and phosphatidylinositol containing structures of malaria parasites. Beta-2-glycoprotein I is a serumprotein known to bind in general to negatively charged phospholipids. By linking sequences of this glycoprotein to the non-dendritic peptide carrier, binding to phospholipids can be mediated, whereby specific targeting of any non-dendritic

peptide carrier derivatized with a disease preventing or curring agent can be achieved.

The following peptides were synthesized:

beta-2-glycoprotein I peptide aa268-278:

KNGMLKGDKVS

This peptide binds antibodies against phospholipids and is, therefore, also relevant in connection with autoimmune diseases,

beta-2-glycoprotein I peptide aa 281-288:

CKNKEKKC

This peptide binds phospholipids.

Peptide alone and the non-dendritic peptide carrier derivatized with peptide were tested in 3 *in vivo* assays for their protective effect against sepsis-like disease manifestations. The testing involves measurement of TNF (tumour necrosis factor) levels.

Experimental design

Treatment scheme

8-12 weeks old (C57BLxBALB/c)F1 mice grouped with 2 mice in each group were used.

The peptides were mixed with the stimulating toxins (10 μg lipopolysaccharide), and the mixture was injected i.p. into the mice.

For TNF assays, priming of mice with BCG was performed 7-10 days before injection of stimulating toxins.

Blood collection

10 µl tail-blood were collected as EDTA plasma, and the following bleedings were performed for the TNF assay:

0, 1, 2, and 4 hours after.

Cell viability

Cell viability was evaluated by tryphaneblue exclusion.

TNF ELISA assays

Murine TNF-alpha levels in sera were measured by a commercial ELISA method performing according to the instructions of the manufacturer (Genzyme, Cambridge, MA).

Results

The non-dendritic peptide carriers decrease lipopolysaccharide toxicity in mice as measured by tumour necrosis factor concentrations in murine sera.

The non-dendritic peptide carrier derivatized with CKNKEKKC peptide or the KNGMLKGDKVS peptide inhibited TNF secretion *in vivo* in mice induced by lipopolysaccharides, while the control peptide alone caused no blocking (Fig. 23).

Other diseases

Other therapeutical uses of the non-dendritic peptide carriers according to the present invention include linking of HIV-1 protease inhibitor peptides to the construct or the linking of blood-brain barrier specific peptides such as E-selectin having the amino acid sequence:

THLVAIQNKEEIEYL

or the pertussis toxin, S_2, having the amino acid sequence:

RALTVAELRGSGDLQEYL

See Example 28.

Example 22

Inhibition of malaria antigen induced cytokine secretion by linking a beta-2-glycoprotein I peptide to a non-dendritic peptide carrier

Background

Pathogenicity of *P.falciparum* malaria parasites appears to be associated with the parasites multiplication, cytoadherence to endothelial cells, and their induction of cytokines like TNF-alpha and IL-6. Malaria drugs are designed to block parasite multiplication, but their ability to interfere with parasite cytoadherence or parasite induction of cytokine secretion is rarely investigated although a recent study showed that chloroquine decreased TNF-alpha secretion from human monocytes stimulated by lipopolysaccharide. African children may die even after they have had their parasites eliminated by malarial drugs probably because of inflammatory reactions in the brain. Drug-mediated interference with parasite cytoadherence or parasite induced production of cytokines is likely to reduce mortality and morbidity caused by malaria parasites.

Materials and Methods

Synthetic peptides

beta-2-glycoprotein I peptide aa 281-288:

KNGMLKGDKVS

This peptide binds antibodies against phospholipids and is therefore also relevant in connection with autoimmune diseases.

beta-2-glycoprotein I peptide aa 281-288:

CKNKEKKC

This peptide binds phospholipids.

Malaria parasite cultures

The *P.falciparum* isolate 3D7 was kept in continuous cultures using RPMI 1640 supplemented with 21 mM sodium bicarbonate, 25mM HEPES buffer, and 10% human serum. The parasites were grown in 4% v/v group O positive human erythrocytes.

Exoantigens of *P.falciparum* for stimulation of cytokine production

Exoantigens were affinity purified from culture medium essentially as described previously (Jakobsen et al., 1988), using as a ligand a pool of IgG from clinically immune African adults. Before chromatography, the culture medium was centrifuged at 7000 g for 10 min, filtered through a 0.22 µm membrane, and dialysed overnight at 4°C against column buffer.

Stimulation of cytokine-release from human mononuclear cells

Human peripheral blood mononuclear cells from different Danish donors were suspended in 3% (v/v) human serum in RPMI 1640 and adjusted to $2X10^6$ cells per ml; subsequently, 0.1 ml volumes were dispensed into wells of 96-well microtiter plates. Peptides in two-fold dilutions (0-100 µg/ml) and optimal concentrations of stimulating malaria antigens, diluted in RPMI 1640 with 3% serum, were then added to a total volume of 0.2 ml. per well. Mononuclear cells incubated with stimulating antigens without peptides, and cells incu-

bated with medium alone served as positive and negative controls, respectively. The cultures were incubated overnight for IL-6 and TNF assays.

ELISA for IL-6

Supernatants were collected and assayed for IL-6 by ELISA procedures (Hansen et al., 1991; Jakobsen et al., 1993) with the following modifications. ELISA Maxisorp plates (NUNC, Roskilde, Denmark) were coated for 24 hours at 4°C with 100 µl per well of 2.5 µg/ml rabbit polyclonal IgG to human recombinant IL-6 in 100 mM $NaHCO_3$, pH 9.6. Non-attached sites were blocked for 1 hour by 100 ul per well of 2% human serum albumin in phosphate buffered saline, pH 7.2 (1 hour at 37°C).

The wells were washed 4 times in 2.5% NaCl, 0.1% Tween 20 (Merck, Darmstadt, Germany). This washing procedure was done after each of the following incubation steps:

1) 100 µl culture supernatants made to 50% (v/v) in incubation buffer, (4% (v/v) normal rabbit serum, (DAKO code X902), 1% polyethylene glycol (mol.wt 6000), 2.5% NaCl, 0.2% Tween 20 in phosphate buffered saline) were incubated for 2 hours at 37°C, dilutions of recombinant cytokine standards in the same incubation buffer were assayed in parallel with the tested supernatants.

2) 100 µl per well of biotinylated rabbit antibodies to recombinant IL-6 (1.5 µg/ml in 0.5% human serum albumin, 0.1% Tween 20 in PBS) were added at 37°C for 1½ hours.

100 µl per well of 0.67 mg/ml 1,2-phenyldiamine hydrochloride (DAKO) dissolved in 100 mM citric acid-phosphate buffer, pH 5.0, containing 0.015% (v/v) H_2O_2. The reactions were stopped by adding 50 µl per well of 2.5 M H_2SO_4, and the optical densities were measured in an ELISA scanner at 490 nm against a test reference at 620 nm.

Results

Non-dendritic peptide carriers inhibited malaria parasite toxin activity *in vitro* as measured by secretion of tumour necrosis factor and interleukin-6. The peptides did not affect lipopolysaccharide toxity *in vitro*. The inhibition of malaria parasite toxin activity was not caused by toxity to the cells as cell viability was not affected.

In pilot experiments, the non-dendritic peptide carrier derivatized with the CKNKEKKC peptide inhibited IL-6 secretion induced by malaria antigens, while the control peptide alone caused only minor blocking of IL-6 secretion (Fig. 12). Means of two experiments are shown in the figure.

The non-dendritic peptide carrier derivatized with the CKNKEKKC peptide inhibited both TNF-alpha and IL-6 secretion from human mononuclear cells stimulated with malaria parasite exoantigens (Table 3 below). The control peptide showed no blocking effect. None of the peptides blocked lipopolysaccharide induced cytokine secretion (Table 4 below). In addition, none of the peptides affected cell viability (Table 5 below), indicating that the peptide constructs have no or low toxity.

Table 3

Percentage inhibition of malaria exoantigen induced TNF-alpha and IL-6 secretion from human peripheral mononuclear cells *in vitro* by a derivatized non-dendritic peptide carrier (NDPC) derivatized with peptide CKNKEKKC or KNGMLKGDKVS, and by a control peptide-CKNKEKKC. Means and standard deviations are shown.

Table 3		
Peptide (µg/ml)	**TNF-alpha**	**IL-6**
CKNKEKKC (NDPC)	n=7	n=7
200	61.9% (28.6)	55.5% (33.2)
100	47.5% (27.4)	34.4% (41.2)
50	50.0% (36.5)	40.8% (38.8)
25	34.2% (27.7)	24.8% (37.0)
CKNKEKKC (control)	n=7	n=7
200	19.6% (15.7)	0.7% (9.4)
100	15.7% (10.3)	-6.2% (6.7)
50	10.7% (36.5)	-2.4% (11.4)
25	12.2% (18.2)	-3.7% (9.4)
KNGMLKGDKVS (NDPC)	n=7	n=7
200	13.9% (19.9)	19.9% (18.8)
100	-8,9% (27.0)	5.3% (6.4)
50	14.0% (25.0)	1.7% (15.2)
25	8 5% (19.4)	-7.6% (7.4)

Table 4

Percentage inhibition of *E.coli* lipopolysaccharide induced TNF-alpha and IL-6 secretion from human peripheral mononuclear cells *in vitro* by a derivatized non-dendritic peptide carrier (NDPC) derivatized with peptide CKNKEKKC or KNGMLKGDKVS, and by a control peptide-CKNKEKKC. Means and standard deviations are shown.

Table 4		
Peptide (µg/ml)	**TNF-alpha**	**IL-6**
CKNKEKKC (NDPC)	n=4	n=4
200	-7.7% (17.5)	-13.0% (6.1)
100	-15.0% (25.1)	-13.0% (3.1)
50	-27.0% (21.7)	-22.5% (7.2)
25	-14.4% (41.7)	-12.9% (4.4)
CKNKEKKC (control)	n=4	n=4
200	-5.6% (7.7)	-6.6% (7.1)
100	3.9% (22.7)	-9.5% (10.2)
50	9.1% (7.5)	-4.7% (2.6)
25	10.9% (20.0)	-2.7% (2.7)
KNGMLKGDKVS (NDPC)	n=4	n=4
200	16.4% (34.3)	2.0% (8.6)
100	2.6% (22.9)	-4.9% (6.4)
50	9.5% (50.6)	-8.5% (7.9)
25	-3.7% (42.5)	-7.7% (7.4)

Table 5

Percentage mononuclear cell viability after incubation with peptides and malaria parasite exoantigens. Means and standard deviations of 3 experiments are shown. Cell viability after antigen stimulation without peptides was 96.3% (1.04), and cell viability after no stimulation was 96.9% (2.95).

Table 5		
CKNKEKKC (NDPC)	**With antigen**	**Without antigen**
200 µg/ml	96.3% (1.99)	96.4% (1.89)
100 µg/ml	97.1% (1.45)	97.8% (1.43)
50 µg/ml	94.6% (1.01)	96.7% (0.71)
25 µg/ml	94.5% (3.26)	96.9% (3.26)
CKNKEKKC (control)	**With antigen**	**Without antigen**
200 µg/ml	98.0% (1.45)	96.5% (0,72)
100 µg/ml	96.8% (1.81)	97.3% (0,97)
50 µg/ml	97.2% (1.91)	96.0% (2.50)
25 µg/ml	97.5% (1.67)	98.2% (1.78)
KNGMLKGDKVS (NDPC)	**With antigen**	**Without antigen**
200 µg/ml	96.4% (2.76)	95.6% (3.01)
100 µg/ml	97.4% (1.85)	96.9% (1.00)
50 µg/ml	96.7% (1.98)	95.2% (2.29)
25 µg/ml	97.4% (1.44)	96.1% (1.75)

Example 23

Down regulation of the immune system in connection with autoimmune diseases by the non-dendritic peptide carriers

The non-dendritic peptide carrier may include immunogenic peptides in combination with bioactive peptides for specific downregulation of immune responses against any immunogenic agent. Alternatively, the non-dendritic peptide carrier derivatized with immunogenic peptides can be used in combination with immunosuppressive cytokines, like IL-10 and TGF-beta, for specific downregulation of immune responses against any immunogenic agent. Thus, tolerance to a specific peptide or protein fragment may be induced. This strategy may be used for protection against or therapy for toxic diseases, autoimmune diseases (diabetes, arthritis, sclerosis etc.).

One example of the modulating effect is given below. Mice were immunized with the non-dendritic peptide carrier derivatized with the malaria EBA-175 peptide three times, inducing high antibody reactivities against the peptides. The non-dendritic peptide carrier derivatized with the EBA-175 peptide was then mixed with murine IL-10, and immunization was performed the fourth time, followed by bleeding and screening for peptide specific antibodies.

Materials and methods

Mice were immunized with non-dendritic peptide carrier constructs containing one model-peptide:

TLTKEYEDIVLKSHMNRESDD

This peptide is derived from the malaria parasite *Plasmodium falciparum*. The peptide covers a sequence of the erythrocyte binding antigen-175 (EBA-175), involved in the parasite invasion of erythrocytes. However, the sequence is normally not recognized by the immune system during infections.

Female 6-8 weeks old (C57BIxBALB/c)F1 mice were used in these studies.

Mice were immunized with the non-dendritic peptide carrier derivatized with EBA-175 peptide. Some mice were immunized subcutaneously 3 times, day 0, 21, and 49, with 16 μg of peptide constructs mixed 1+1 with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant. Mice were immunized a fourth time, day 70, subcutaneously with the peptide-construct mixed with Freund's incomplete adjuvant with and without 1 μg of murine recombinant IL-10.

The mice were bled on days -1, 12, 33, 61, and 82. Sera were collected from the bleedings and tested in ELISA for antibody reactivity against the control EBA-175 peptide.

The ELISA was performed as described in Example 20 in the patent application.

Results:

Eight mice responding to EBA-175 peptide with Freund's complete adjuvant were immunized one more time (4th immunization). Four mice received non-dendritic peptide carriers derivatized with EBA-175 peptides plus Freund's incomplete adjuvant, and four mice received the same plus murine recombinant IL-10, a downregulator of the immune system.

Fig. 24 shows that mice immunized with the peptide-adjuvant combination alone had a stable or slight decline in antibody reactivity, while mice immunized with the peptide-adjuvant combination plus IL-10 showed a marked decrease in antibody reactivity.

In addition, the non-dendritic peptide carrier constructs may be used in general to prolong the presence in circulation of any medicament such as peptide drugs.

Example 24

Non-dendritic peptide carriers derivatized with immunogenic peptides for specific induction of Th1-like and Th2-like responses

The non-dendritic peptide carriers derivatized with immunogenic peptides can be used in combination with cytokines for specific induction of Th1-like and Th2-like cellular responses against any immunogenic agent. In connection with specific induction of such responses, the non-dendritic peptide carrier in combination with cytokines can thus be used for vaccination purposes to protect against pathogenic microorganisms, for therapy against infectious diseases, autoimmune diseases. Specific induction of Th1-like responses may be used for protection against diseases like leishmaniasis, tuberculosis, and possibly AIDS, and for therapy against allergic diseases etc., whereas specific induction of Th2-like responses may be used for protection against worm diseases and for therapy against toxic diseases (sepsis, meningitis, etc.).

Examples of the regulation of the immune system by non-dendritic peptide carriers are given below in which mice are immunized with a synthetic Human Immunodeficiency Virus-1 specific peptide or with *Leishmania major* specific peptides.

Materials and methods:

Mice were immunized with non-dendritic peptide carriers derivatized with one or more of the different model-peptides:

The HIV-1 peptide gp120,

aa152-176, (GEIKNCSFNISTSIRGKVQKEYAFF),

with known B- and T-cell epitopes.

L1: YDQLVTRVVTHEMAHA

This Leishmania specific peptide is reported to contain a T-cell epitope.

L2: EAEEAARLQA (H-2^{d} restricted Th2 epitope in Balb/C mice).

Cytokine peptides:
The IFN-gamma bioactive sequences:
IFN-gamma (1-39)
HGTVIESLESNNYFNSSGIDVEEKSLFLDIWRNWQKDG
IFN-gamma (95-133)
AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC

The TNF bioactive sequence (TNF70-80):
Pro-Ser-Thr-His-Val-Leu-Ile-Thr-His-Thr-Ile

Tuftsin peptide:
TKPR

Recombinant murine IFN-gamma, IL-4, IL-12, and TNF-alpha were purchased.

Synthetic peptide sequences of or recombinant gamma-interferon, IL-12 or IL-18 may be used for induction of Th1 responses. Synthetic peptide sequences of or recombinant IL-4, IL-5, or IL-13 may be used for induction of Th2 responses. Addition of adhesion molecules like B7-1, B7-2, P-selectin, or E-selectin may also affect the induction of Th1 and Th2 responses. The peptides and/or the cytokines and/or adhesion molecules may be used alone or in vehicles, with adjuvants, inserted into immunostimulating complexes, liposomes etc.

The dose of peptide as well as addition of different antigen presenting cells may also affect the induction of Th1 and Th2 responses.

Experimental procedure

Female 6-8 weeks old BALB/cJ mice (reported to be a Th2 responder mouse) were used in these studies. Mice were immunized with the non-dendritic peptide carriers derivatized with Leishmania or HIV-1 peptides without any conjugations. Some mice were immunized intraperitoneally 3 times, day 0, 21, and 49, with 16 μg of peptide constructs, while some other mice were immunized subcutaneously 3 times, day 0, 21, and 49, with 16 μg of peptide constructs alone or mixed 1+1 with alum, with alum and recombinant cytokines or with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant.

The mice were bled on days -1, 12, 33, and 61. Sera were collected from the bleedings and tested in ELISA for IgG_{2a} (marker of Th1) and IgG1 (marker of Th2) antibody reactivity against the leishmania peptide or the HIV-1 gp-120 peptide.

The ELISA was performed as described in Example 20 in the patent application.

Peripheral mononuclear cells, lymph node cells, and spleen cells will be collected and stimulated *in vitro* with the peptide, recombinant gp120 or PPD in optimal concentrations. After 5-7 days of incubation, the supernatants will be harvested and tested for their content of gamma-interferon (as an indicator of Th1 responses) and for their content of interleukin-4 (as an indicator of Th2 responses), using commercial ELISA-kits according to the instructions of the manufacturer. Quantification of gamma-interferon mRNA and interleukin-4 mRNA within the cells may also be undertaken.

Results

Immunization experiments with the leishmania peptide L1: Mice were immunized with the non-dendritic peptide carrier deriva-

tized with L1 peptide plus alum alone or with Freund's complete adjuvant or with alum and one of the following recombinant murine cytokines: interferon-gamma, tumour necrosis factor (TNF), IL-12 or IL-4.

Fig. 25 shows the IgG1 response (putative Th2 response) of mice immunized with these L1 combinations. All mice produced IgG1 after 3 immunizations. Maximum responses were detectable after immunizations with recombinant TNF, recombinant IFN-gamma or Freund's complete adjuvant.

Mice immunized with recombinant TNF or recombinant IL-12 responded after 2 immunizations.

Fig. 26 shows the IgG2a response (putative Th1 response) of mice immunized with the same L1 combinations.

Mice immunized with recombinant TNF or Freund's complete adjuvant responded after 3 immunizations. Weak responses were detectable with the other L1 combinations.

A biphasic IgG2a response were recorded. Mice immunized with most of the combinations showed an IgG2a response after one immunization.

Conclusion:

Mice immunized with non-dendritic peptide carriers derivatized with L1 peptide and recombinant cytokines showed a strong antibody response to L1 peptide. All mice showed a strong Th2 like IgG1 response after 3 immunizations although many of them were immunized with a Th1 cytokine. The implications are that IgG1 and IgG2a may not be reliable markers of Th2 and Th1 responses, or that Th1 cytokines may also induce a Th2 response after repeated exposure. Th1 responses have been reported to develop before Th2 responses during experimental infections. We recorded a transient IgG2a response (putative Th1 response) after a single immunization. Some of these mice

did not show an IgG2a response after repeated immunizations. The strong IgG2a response detectable in two of the groups after 3 immunizations may be very different from the IgG2a response recorded after 1 immunization.

It was a novel finding that a synthetic peptide combination could be immunogenic after a single immunization. Such findings have only been recorded for DNA vaccines and attenuated microorganisms. An IgG2a response, after a single immunization, may be a marker of a Th1 response.

Mice were also immunized sc. and ip. with non-dendritic peptide carrier derivatized with L1 peptide and tuftsin. Mice showed a strong IgG1 response to L1-tuftsin construct sc. and a moderate response to L1-tuftsin construct ip. and to the L1 constuct alone sc. (fig. 27). The L1-tuftsin combinations did not induce an IgG2a response (fig. 28). Tuftsin is therefore interesting in that it only induces one branch of the immune system.

Mice were also immunized with non-dendritic peptide carriers derivatized with peptides covering 2 different sequences of IFN-gamma reported to mediate binding to the IFN-gamma receptor. Different combinations were immunogenic after 3 immunizations sc. (Fig. 29). Immunization with L1 and both IFN-gamma peptides induced the strongest IgG1 response, and this combination was immunogenic after 2 immunizations. This combination was also capable of inducing an IgG2a response after 3 immunizations (Fig. 30).

The different IFN-gamma peptides also induced a relatively weak IgG1 response after ip. immunizations (Fig. 31), but they did not induce an IgG2a response (Fig. 32).

Finally, mice were also immunized with non-dendritic peptide carriers derivatized with L1 peptide together with a peptide covering a sequence of TNF. The L1-TNF-peptide combination sc. induced a strong IgG1 response (Fig. 33), while ip.

immunizations did not induce an IgG1 response (data not shown).

The sc. immunization with non-dendritic peptide carrier derivatized with the L1-TNF peptide combination also induced a biphasic IgG2a response comparable to the non-dendritic peptide carrier derivatized with L1 peptide mixed with recombinant TNF, while ip. immunizations did not induce an IgG2a response (Fig. 34).

The reproducibility of the results were tested with another Leishmania peptide, L2, reported to be a Th2 inducing peptide. The non-dendritic peptide carrier derivatized with L2 peptide alone or combined with tuftsin or with the TNF peptide all induced an IgG1 response after sc. immunizations, while ip. immunizations were not immunogenic (Fig. 35). The sc. immunizations did not induce an IgG2a response, but ip. immunizations with the non-dendritic peptide carriers derivatized with L2-tuftsin or L2-TNF-peptide combinations ip. induced an IgG2a response after one immunization, but not after 3 immunizations (Fig. 36).

Non-dendritic peptide carriers derivatized with the two IFN-gamma peptides in combination, tuftsin or the TNF peptide, were also tested in combination with the HIV-1 gp120 peptide. Immunizations sc. stimulated IgG1 production with the TNF peptide containing construct being the most immunogenic (fig. 37). Immunizations ip. were not immunogenic.

Immunizations sc. with non-dendritic peptide carriers derivatized with the IFN peptides or the TNF peptide induced a biphasic IgG2a response with responses detectable after the first and the third immunization (Fig. 38). Immunizations ip. were immunogenic after one immunization, but not after 3 immunizations. Tuftsin containing constructs did not induce an IgG2a response.

Conclusion:

Several synthetic peptide combinations induced strong antibody responses without use of adjuvants or carriers. The fact that so many different antigenic peptide cytokine peptide combinations are strong immunogens is an entirely new finding.

Example 25

Non-dendritic peptide carriers derivatized with immunogenic peptides for specific induction of enhanced cellular responses

Background:

The non-dendritic peptide carriers may include immunogenic peptides in combination with bioactive cytokine peptides for specific induction of enhanced cellular responses against any immunogenic agent.

Alternatively, the non-dendritic peptide carrier derivatized with immunogenic peptides can be used in combination with cytokine molecules for specific induction of enhanced cellular responses against any immunogenic agent. The non-dendritic peptide carrier in combination with cytokines can thus be used for vaccination purposes to protect against pathogenic microorganisms, for therapy against infectious diseases, toxic diseases, autoimmune diseases, etc.

One example of this use of the non-dendritic peptide carrier is given below in which mice were immunized with the non-dendritic peptide carrier derivatized with a synthetic Human Immunodeficiency Virus-1 specific peptide and with a single copy of an IL-1 beta peptide or a tuftsin peptide included in the structure.

Materials and methods:

An IL-1 beta bioactive sequence:

VQGEESNDK/Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys

or the TNF bioactive sequence:

Pro-Ser-Thr-His-Val-Leu-Ile-Thr-His-Thr-Ile

or the tuftsin sequence:

TKPR,

were synthesized N- or C-terminally within the non-dendritic peptide carrier derivatized with the peptide from HIV-1 gp120:

aa152-176: GEIKNCSFNISTSIRGKVQKEYAFF

with known B- and T-cell epitopes.

The non-dendritic peptide carrier construct may be used alone, after conjugation to PPD, after insertion into immunostimulating complexes or liposomes, after addition of recombinant cytokines, adhesion molecules, adjuvants, vehicles, carriers etc.

The same experimental and testing procedure as described in Example 20 above was followed.

Female 6-8 weeks old (C57BIxBALB/c)F1 mice were used in these studies.

Mice were immunized with the non-dendritic peptide carriers derivatized with the HIV-1 peptide with and without the IL-1 or tuftsin sequences without any conjugations. Mice were immunized subcutaneously 4 times, day 0, 21, 49, and 70, with

16 µg of peptide constructs alone or mixed 1+1 with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant.

The mice were bled on days -1, 12, 33, 61, and 82. Sera were collected from the bleedings and tested in ELISA for antibody reactivity against the linear HIV-1 gp-120 peptide.

The ELISA was performed as described in Example 20 in the patent application.

Results:

Experiments indicate that the non-dendritic peptide carrier derivatized with the HIV-1 gp120 peptide alone or with the inclusion of an IL-1 beta specific peptide sequence or the tuftsin sequence is immunogenic without the use of adjuvant. The non-dendritic peptide carrier structure may be acceptable for use in humans and provide a strong immunogenic stimulus to the immune system with applications in vaccine technology.

The non-dendritic peptide carrier derivatized with the gp-120 peptide (batch II) alone, in combination with Freund's adjuvant, in combination with the IL-1 peptide or in combination with the tuftsin peptide, induced antibody production after 3 immunizations sc. (Fig. 39). The strongest stimulator was the gp120 peptide plus tuftsin construct.

The non-dendritic peptide carrier derivatized with the gp120 peptide (batch IV), alone or in combination with the IL-1 peptide, induced antibody production after 3 immunizations sc. (Fig. 40).

Example 26

Non-dendritic peptide carriers for use as a therapeutic agent in therapy of cancer.

Non-dendritic peptide carriers may be used to cure or to inhibit cancers by direct neutralization of cancer cell growth and/or metastasis and by inducing neutralizing immune responses to the cancer cells.

To illustrate this application of the invention, examples are given in the following:

Materials and methods.

The following synthetic peptides will be synthesized:

Wildtype p53 peptide: KYMCNSSCM,

Mutant p53 peptide: KYICNSSCM,

and peptides from proteins such as MAGE, BAGE, GAGE, MART, melan, and tyrosinase or a 43 kDa protein, including

QDLTMKYQIF from the kDa protein, including
AAGIGILTV and ILTVILGVL from the MART-1/Melan-A protein
SAYGEPRKL from MAGE-1
SEIWRDIDF from tyrosinase
YRPRPRRY from GAGE-1
and the melanoma peptide YLEPGPVTA

The peptides will be presented as control peptides or in derivatized non-dendritic peptide carrier constructs with or without cytokines (TNF-alpha, GM-CSF), cytokine peptides or adhesion molecules such as B7 or B7 ligands CD28 or CTLA-4.

Female BALB/c mice (age 10-16 weeks) will be used. Mice will be immunized with 36 μg of peptide constructs, 1-3 times.

Tcyt activities:
3×10^6 immune spleen cells will be restimulated *in vitro* for 5-7 days with the peptides (app. 5 μM).

Cytolytic activity of the restimulated cells will be measured by mixing the spleen cells with ^{51}Cr-labelled targets in duplicate and incubated at 37°C for 4 hours. Supernatants will be harvested with a harvesting system and radioactivity will be counted in a gamma counter.

Percent-specific lysis will be calculated using the equation: 100 x ((experimental release-spontaneous release)/(maximal release-spontaneous release)).

Protection against tumours:
Mice (5 per group) will be immunized with non-dendritic peptide carrier constructs and challenged i.v. or s.c. with cancer cells (1-9 x 10^5 sarcoma cells or mastocytoma cells). Tumour growth will be monitored every 7 hours for at least 21 days.

Integrin adhesion molecule sequences and polymers of the RGD sequence will be inserted into the non-dendritic peptide carrier structure and tested for their ability to block melanoma cell metastasis in BALB/c mice.

Cancer-cell specific sequences or carbohydrate structures such as Le^x will be inserted into the non-dendritic peptide carrier structure and used for diagnostical identification of micro metastases, using ELISA methodologies as described in Example 19.

Example 27

Non-dendritic peptide carriers against autoimmune diseases: Protection, treatment, and diagnosis

Downregulation of the immune system in connection with e.g. autoimmune diseases may be advantageously performed by administrating relevant peptides linked to the non-dendritic peptide carrier.

One example of this use for treatment of rheumatoid arthritis is the preparation of a therapeutic composition for oral or systemic intake in which the peptide *Mycobacterium bovis* HSP60 aa 180-188 related to arthritis, having the sequence:

TFGLQLELT

or the peptide of HLA and DnaJ HSP having the sequence:

Q(K/R)RAA,

is linked to the non-dendritic peptide carrier.

Inbred Lewis rats and Fisher rats will be used. All rats will be of 6-8 weeks of age. Arthritis will be induced by injection of *Mycobacterium tuberculosis* in Freund's complete adjuvant. Rats will be injected intracutaneously at the base of the tail with 100 µl of 10 mg/ml of *M.tuberculosis*. The rats will be observed for clinical arthritis and scored by grading of each paw from 0 to 4 based on erythema, swelling, and deformity of the joint. All four legs will be scored, so the highest score achievable will be 16.

Rats will be immunized 1-3 times intraperitoneally or subcutaneously with 5.35 and 200 µg of peptide constructs (plus cytokine sequences and other immunomodulatory sequences in some experiments) app. 35 days before induction of arthritis, and scores for clinical arthritis will be obtained. Lymph node lymphocytes will be collected 9 days after immunization, and the lymphocytes will be tested for *in vitro* proliferation, IFN-gamma, and IL-4 production, in response to *M.tuberculosis* and to the peptide constructs.

Immunological responses to the peptide constructs will be measured by testing for murine subclass Ig reactivities to the peptides.

Another example is the linking of various peptides relevant in connection with encephalomyelitis (sclerosis) to the non-dendritic peptide carrier. Examples of such peptides include:

Rat T-cell receptor, having the peptide sequence:

DMGHGLRLIHYSYDDVNSTEKG,

rat T-cell receptor, having the peptide sequence:

ASSDSGNTE, and

the myelin basic protein Ac1-11: AcASQKRPSQRSK, where "Ac" denotes an acetyl group,

and the myelin basic protein p87-99: VHFFKNIVTPRTP with and without amino acid substitutions, and

NTWTTCQSIAFPSK or SKTSASIGSLCADARMYGVL or LINVIHAFQYV from the myelin proteolipid protein and PGYPIRALVGD from the myelin oligodendrocyte glycoprotein.

Experimental procedure:

Female Lewis rats, 6-12 weeks old, or (PL/JxSJL/J)F_1 mice or BALB/c mice or CBA mice or Biozzi ABH mice will be used.

Rats will be immunized 1-3 times intraperitoneally or subcutaneously with 5.35 and 200 µg of peptide constructs (plus cytokine sequences and other immunomodulatory sequences in some experiments) app. 35 days before or together with Freund's incomplete adjuvant supplemented with heat-killed 1 mg *Mycobacterium tuberculosis* (1+1) sc. At 30 days, each rat will be challenged with myelin basic protein (50 µg in complete Freund's adjuvant or similar proteins). Rats will be monitored daily from day 9 for clinical signs (tail weakness and limb paralysis). Alternatively, the rats may be fed with peptide constructs 2 days before challenge.

Lymph node lymphocytes will be collected 9 days after immunization, and the lymphocytes will be tested for *in vitro* proliferation, IFN-gamma, and IL-4 production, in response to *M.tuberculosis* and to the peptide constructs.

Immunological responses to the peptide constructs will be measured by testing for murine subclass Ig reactivities to the peptide constructs.

Another example is the linking of various peptides relevant in connection with diabetes to the non-dendritic peptide carrier. Examples of such peptides include:

Pseudomonas HSP60 peptides:
- VLGGGCALLRCIPACDSTLPANED
- PALDSLTPANEA

Glutamic acid decarboxylase peptidE $GAD65_{253-265}$: IARFKMFPEVKEK

Glutamic acid decarboxylase peptidE $GAD65_{524-543}$: SRLSKVAPVIKARMMEYGTT.

HLA peptides TPQGRP(V/A/D/S)AEY or peptides containing this sequence,

and the regulatory peptide:
cks17:LQNRRGLDLLFLKEGGL.

Studies will be performed in Non-Obese Diabetic mice (NOD mice), 3-22 weeks old, which develop diabetes. NOD mice will be immunized s.c., i.p., orally or by nasal administration, 1-3 times (at weeks 3, 7, and 10) with peptide constructs (5-500 μg) alone or in combination with tuftsin or cytokines (peptides and recombinant proteins).

Immunological responses to the peptides will be measured by testing for murine subclass Ig reactivities to the peptides

and by testing lymph node and spleen cell proliferation and gamma-interferon and interleukin-4 production *in vitro* in response to peptide stimulation. Development of diabetes after app. 12 weeks in the vaccinated mice will be monitored.

For assessment of diabetes, mice will be sacrificed and pancreata and salivary glands will be fixed in formalin, embedded in paraffin, and stained with hematoxylin/eosin and scored for islet pathology. Mice will be tested weekly for glucosuria by a commercial kit. Blood glucose will be measured by using a blood glucose meter.

All such non-dendritic peptide carriers may be given in combination with any immunomodulator or part thereof having immunomodulator activity. Relevant immunomodulators include interleukin-4, interleukin-10, and TGF-beta.

Another example is the linking of various peptides relevant in connection with hen's egg allergy to the non-dendritic peptide carrier. Examples of such peptides include:

EFRADHPFLF or peptides including this sequence from ovalbumin inhibition of histamine release from basophils:

Whole blood (50 µl) diluted 1:2 in N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (20 mmol/l)-Tyrode's buffer will be mixed at 4°C for 24 hours with 40 µl of peptide constructs at concentrations 0-28,000 µg/ml dissolved in the same buffer. Blood pretreated with peptides will be challenged for 30 minutes at 37°C with 50 µl of various dilutions of allergen. The reaction will be stopped by centrifugation at 1,500*g* at 4°C for 5 minutes. Histamine will be measured by an Enzyme Linked Immunosorbent Assay or a radioimmunoassay. The assays will be performed in duplicate. Spontaneous histamine release from the cells will be subtracted from the calculated histamine release. Percent inhibition of histamine release by peptide constructs will be measured.

Example 28

The use of derivatized non-dendritic peptide carriers (DNDPC) for increasing blood-brain barrier permeability to peptides, drugs, diagnostic markers and other substances

Non-dendritic carrier peptides may be used to facilitate blood-brain barrier transfer for the purpose of brain and neurological therapy and diagnostics by intravenous (iv.) administration; this is illustrated in the following two planned examples.

A blood-brain barrier decreasing substance, including N-acetylglucosamine-N-acetyl muramic acid-Ala-Glu-Dap-Ala, where Dap is diaminopimelinic acid, (Spellerberg 1995), will be coupled as branch-moieties to an NDPC of the standard design described in Table 1.

Blood-brain barrier permeabilization will be analyzed by iv injection of FITC-dextran of 4, 10, 20, and 50 kD at fixed time points after iv injection of the DNDPC in rabbits as described (Spellerberg 1995). After anaestization cerebrospinal fluid will be collected and analyzed for fluorescence.

It is expected that the DNDPC will transiently increase BBB-permeability for molecules below 20 kD with a maximum permeability around 4 hours after DNDPC-administration.

In another experiment, it will be shown that a DNDPC is efficient in enhancing peptide-specific transport through the BBB of the DNDPC itself. The DNDPC will be prepared with multiple copies of one type of attached moiety, selected from

- YGGFM
- YGGFL
- Lys_N, in which N = 3-8

and another type of attached moiety, which in this example will be a biotin-labelled model peptide:

biotin-LKYGGENKPGG.

In the planned experiment, rabbits will be injected iv., and the cerebrospinal fluid will subsequently be analyzed for avidinbinding by ELISA.

In all cases, the presence of biotin-peptide is expected to increase when biotin-peptide is administered as a part of a DNDPC compared to when administered alone.

Example 29

The use of derivatized non-dendritic peptide carriers (DNDPC) to inhibit cell-matrix attachment

This example demonstrates a therapeutic use of a derivatized non-dendritic peptide carrier to stop the spread of cancerous cells by inhibiting their matrix attachment. By including cell-matrix binding interfering peptides as attachment peptides in a DNDPC, a very efficient inhibitory substance is expected to be obtained. In a planned experiment, peptides containing sequences selected from
RGDS
RLDS
YIGSR
and multimers thereof (N = 2-4)(Fujii 1995, Nomizu 1993) will be attached to an NDPC and tested for *in vivo* inhibition of tumor growth and metastasis as compared to the corresponding free peptides, using the experimental protocols detailed in Nomizu et al. (1993) or equivalent protocols.

In short, for judgement of metastasis, mice will be injected with detached melanoma cells in the presence or absence of peptide by the tail vein, and, around 2½ weeks later, mice will be sacrified and the number of lung colonies will be

enumerated. For tumor growth, the same cells will be administered subcutaneously, followed by daily i.p. injections of peptides. On day 10, mice will be sacrificed and the subcutaneous tumors will be weighed.

Example 30

The use of derivatized non-dendritic peptide carriers (DNDPC) containing carbohydrate antigen mimotopes for the production of carbohydrate-specific antibodies for diagnosis and vaccination; application to the Tn-antigen

Peptides that mimick carbohydrate epitopes (peptide mimotopes) can be defined by peptide library scanning methods with suitable monoclonal antibodies against the carbohydrate epitope in question. Such a mimotope peptide is expected to be of use as an immunogen in the active immunotherapy of cancer and HIV, and it is also expected to be a better immunogen, especially with respect to induction of immunological memory than the corresponding carbohydrate antigen.

The Tn-antigen (N-acetyl-D-galactosamine on serine or threonine), which is associated with cancer and with HIV-infection, and a mimotope corresponding to the Tn-carbohydrate antigen are planned to be employed in the following illustrative way:

The Tn-mimotope peptide will be synthesized as an attached antigenic peptide on a non-dendritic peptide carrier (NDPC) as detailed in Example 1, using a RINK-MBHA solid phase. The liberated whole complex will be used for immunizations of mice and rabbits following normal immunization schemes (see Example 20) and using Freund's incomplete adjuvant. In addition, derivatized NDPC in which is included or which is administered together with a stimulating sequence, including a tuftsin tetramer, fragments of cytokines or T-cell stimulatory peptides, may be used for immunizations without the presence of adjuvants as described in Example 20. The result-

ing antibody response is analyzed for specificity against the immunizing peptide *and* the Tn-epitope itself. The Tn-epitope will be obtained from ovine submaxillary mucin which is obtained from Sigma and then desialylated.

Good antibody reactivity is expected to be obtained against the Tn-antigen, and it is also expected that these Tn-specific, peptide-directed antibodies will be able to block HIV-infection and syncytium formation *in vitro*.

Example 31

The use of antibodies against derivatized non-dendritic peptide carriers (DNDPC) in an immunohistochemical diagnostic assay for the prion disease bovine spongiform encephalitis

The prion diseases are characterized by the presence of misfolded prion proteins, presumably caused to misfold by the influence of exogenous misfolded prion proteins. It has been shown in sheep that abnormal prion proteins may be detected pre-clinically by immunohistochemistry on tonsil biopsies from scrapie-infected sheep (Schreuder 1996), using antibodies raised against synthetic peptides corresponding to abnormal prion protein sequences.

To develop the same type of diagnostic antibodies for the bovine prion disease, the prion peptides will be synthesized on a non-dendritic peptide carrier of the standard design described in Example 1 and subsequently used for the production of diagnostic antibodies in rabbits and mice by coinjection with adjuvant in a normal immunization scheme (see Example 20). In addition, derivatized NDPC in which is included or which is administered together with a stimulating sequence, such as a tuftsin tetramer, fragments of cytokines or T-cell stimulatory peptides, may be used for immunizations without the presence of adjuvants as described in Example 20. Each of the following prion peptides will be attached to a non-dendritic peptide carrier, preferably by direct chemical

synthesis, in order to construct 5 different immunization DNDPCs:

GQGGTHGQWNKP
GQWNKPSKPKTN
GGLGGYMLGSAMSRPLIH
GSDYEDRYYRENMHRYPNQVYRPVDQYSNQNN
RESQAYYQRGAS

Antibodies produced against these DNDPCs will be tested on tonsil biopsies from normal cattle and from cattle in a preclinical state of spongiform encephalitis.

Shortly, tonsil biopsies will be pretreated with formic acid and hydrated autoclaving and will then be incubated with the antisera diluted in PBS followed by detection antibodies and staining (as detailed by van Keulen 1995). This treatment selectively enhances the immunoreactivity of the BSE-specific form of the prion protein.

Affected animals are expected to show antibody-positive biopsies before showing any clinical signs of the disease.

Example 32

Immunization against tuberculosis

The following synthetic peptides from the ESAT-6 antigen (Brandt, 1996) will be synthesized:

YQGVGGKWDATATELNNALQ

and

MTEQQWNFAGIEAAASAIQG

as control peptides and derivatized to the non-dendritic peptide carrier.

The peptides will be used for immunization of mice against *Mycobacterium tuberculosis*.

Experimental procedure:

Mice:
C57BL/6J female mice, 8-12 weeks old, will be used.

Bacteria:
M.tuberculosis H37Rv will be grown at 37°C on Lowenstein-Jensen medium or in suspension in Sauton medium enriched with 0.5% sodium pyrovate and 0,5% glucose.

Vaccination challenge and necropsy:
The mice will be immunized 3 times s.c. or i.p. with optimal concentrations of synthetic peptide constructs with and without 2 μg recombinant IL-12. After 12-14 weeks, the mice will be challenged by an i.v. injection of 5×10^4 colony forming units of *M.tuberculosis* suspended in PBS. The course of disease will be compared with that for a corresponding group of unimmunized mice during a period of 28 days. Enumerations of bacteria in the spleen of infected mice will be done by plating double serial 10-fold dilutions of spleen homogenated on Lowenstein-Jensen medium. Colonies will be counted after 3-4 weeks of incubation.

Lymphocyte cultures:
Spleen cells or lymph node cells will be isolated 2-3 weeks after the previous immunization. And activated *in vitro* with peptides or *M.tuberculosis* antigens. Cellular proliferation will be investigated by pulsing cultures with tritiated thymidine after 48 hours of incubation and then further incubating for 22 hours, before the plates will be harvested and processed for liquid scintillation counting.

Culture supernatants will be harvested from parallel cultures after 24 hours, of incubation for determination of IL-2, IL-

4, and IL-5, and, after 48 hours, for determination of interferon-gamma.

Cytokine assays:
The amounts of cytokines present in culture supernatants will be quantified by commercially available ELISA kits.

Antibody titers of different IgG isotypes:
Serum samples will be collected after each immunization and analyzed in two-fold dilutions on ELISA plates coated with peptides or *M.tuberculosis* antigens. Reactivities will be tested with peroxidase labelled goat anti-mouse IgG or anti-mouse IgG1 or anti-mouse IgG2a according to the ELISA procedures described in Example 20.

Detection of cytokine mRNA:
Lymph node cells (10^6) will be lysed and total RNA purified. RNA will be reversely transcribed into cDNA and subjected to PCR amplification with specific primers for individual cytokines. The PCR products will be hybridized with specific labelled oligonucleotide probes for the cytokines in southern blots.

Example 33

The use of a derivatized non-dendritic peptide carrier for the production of antibodies against outer membrane proteins in *Actinobacillus pleuropneumoniae*

Mice were immunized with a derivatized non-dendritic peptide carrier corresponding to type no. 2 and 3 in Table 1, carrying as the branch peptide

AELGGQFHHKSENG (Tbp peptide 4)

from transferrin-binding protein type 2 from *Actinobacillus pleuropneumoniae*, and another group of mice were immunized

with a derivatized non-dendritic peptide carrier corresponding to type 3 in Table 1 carrying as the branch peptide

TEADYAKNRAVLEY (PalA peptide 5)

from proteoglycan-associated protein from the same bacterium.

The peptides were used either alone or in combination with Freund's incomplete adjuvant. Subcutaneous injections were used.

The mice were immunized 3 times at intervals of 14 days. Bleedings were performed before the first immunization (0) and 10 days after each immunization (1, 2, 3).

For comparison, another two groups of mice were immunized with the corresponding peptides conjugated by SPDP through cysteine to PPD ("Purified Protein Derivative") subsequent to a BCG-priming as an example of a classical peptide-protein conjugate immunization method.

The bleedings were analysed by ELISA using the various immunization peptides as coating antigen. Peptides were coated overnight at 4^0C at 1 ug/ml in PBS on Maxisorp (Nunc) microtitre plates. Subsequent operations were performed at room temperature. After blocking for 1 hour in PBS+1% BSA and wash in PBS + Tween 20 (0.1%), mouse sera were applied 1/200 in the blocking buffer and incubated for 1 hour. Subsequently, plates were washed as above and developed by horse radish peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO) for 1 hour followed by colour development by OPD and peroxide (see Fig. 41 and 42).

The antibodies raised against the PPD-coupled Tbp-peptide 4 were cloned, and the resulting monoclonal antibody was tested for its ability to recognise the Tbp-peptide 4 in different presentations (see Fig. 43) by an indirect ELISA using the different peptides as coating antigens.

Furthermore, reactivity with the proteins was analysed by Western blotting of whole cell extracts of *Actinobacillus pleuropneumoniae* (not shown).

In conclusion, the response against the derivatized non-dendritic peptide carrier with Freund's incomplete adjuvant is similar to the response obtained with PPD-conjugated peptides (see Fig. 41 and 42), and protein-reactive antibodies are obtained by both methods as shown by blotting (not shown). Furthermore, the monoclonal anti-PPD-Tbp-peptide 4 antibody selectively recognizes backbone-conjugated peptides, not free peptides whether in Iscoms or not (Fig. 43). This provides evidence that conjugation to the non-dendritic carrier peptide of this invention stabilizes the conformation of the branch-peptide at least as much as is obtained by classical conjugation methods.

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(iii) NUMBER OF SEQUENCES: 52

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Pro Tyr Lys Cys Pro Glu Cys Gly Lys Ser Phe Ser Gln Lys Ser Asp
1 5 10 15

Leu Val Lys His Gln Arg Thr His Thr Gly
20 25

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Thr Lys Pro Arg
1

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Thr Lys Pro Arg Thr Lys Pro Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg
1 5 10 15

Thr Lys Pro Arg
20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Phe Asn Asn Phe Thr Val Ser Phe Trp Leu His Arg Val Lys Val Ser
1 5 10 15

Ala Ser His Leu Glu
20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Asp Gln Val His Phe Gln Pro Leu Pro Pro Ala Val Val Lys Leu Ser
1 5 10 15

Asp Ala Leu Ile
20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Asp Ile Glu Lys Lys Ile Ala Lys Met Glu Lys Ala Ser Ser Val Phe
1 5 10 15

Asn Val Val Asn Ser
20

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Lys Leu Leu Ser Leu Ile Lys Gly Val Ile Val His Arg Leu Glu Gly
1 5 10 15

Val Glu

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Leu Asp Asn Ile Lys Gly Asn Val Gly Lys Met Glu Asp Tyr Ile Lys
1 5 10 15

Lys Asn Asn Lys
20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Val Ala Lys Leu Glu Ala Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Ala Val His Lys Leu Glu His Lys Val Ala Lys Leu Glu Ala Lys Gly
1 5 10 15

Lys Gly Lys Tyr
20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Leu Gln Gly Gln Asp Met Glu Gln Gln Val

1 5 10

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Asp Pro Lys Asn Tyr Pro Lys Lys Lys Met Glu Lys Arg Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Val Gln Gly Glu Glu Ser Asn Asp Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Gly Gly Thr Lys Gly Gly Gln Asp Ile Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Tyr Gly Leu Ala Glu Leu Lys Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Gly His Pro Leu Gln Lys Thr Tyr
1 5

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Leu Thr Pro Leu Glu Glu Leu Tyr Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Lys Asn Gly Met Leu Lys Gly Asp Lys Val Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Cys Lys Asn Lys Glu Lys Lys Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Leu Glu Arg Leu Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Gly Glu Ile Lys Asn Cys Ser Phe Asn Ile Ser Thr Ser Ile Arg Gly
1 5 10 15

Lys Val Gln Lys Glu Tyr Ala Phe Phe
20 25

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Trp Gly Cys Ser Lys Leu Ile Cys Thr Thr Ala Val Pro Trp Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Leu Gln Asp Gln Ala Arg Leu Asn Ser Trp Gly Cys Ala Phe Arg Gln
1 5 10 15

Val Cys His Thr
20

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Thr Leu Thr Lys Glu Tyr Glu Asp Ile Val Leu Lys Ser His Met Asn
1 5 10 15

Arg Glu Ser Asp Asp
20

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Leu Lys Ser His Met Asn Arg Glu Ser Asp Asp Gly Glu Leu Tyr Asp
1 5 10 15

Glu Asn Ser

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Pro Ser Thr His Val Leu Leu Thr His Thr Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Val Thr Glu Glu Ile
1 5

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Val Thr Glu Glu Ile Val Thr Glu Glu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Val Thr Glu Glu Ile Val Thr Glu Glu Ile Val Thr Glu Glu Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Val Tyr Lys Leu Glu Ala Lys Val Ala Lys Leu Glu Ala Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Cys Met Ser Asp Gly Ala Val Gln Pro Asp Gly Gly Gln Pro Ala Val
1 5 10 15

Arg Asn Glu Arg Ala Thr
20

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Met Ser Asp Gly Ala Val Gln Pro Asp Gly Gly Gln Pro Ala Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

Gln Pro Asp Gly Gly Gln Pro Ala Val Arg Asn Glu Arg Ala Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

Asp Met Gly His Gly Leu Arg Leu Ile His Tyr Ser Tyr Asp Asp Val
1 5 10 15

Asn Ser Thr Glu Lys Gly
20

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

Ala Ser Ser Asp Ser Gly Asn Thr Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Thr Leu Phe Gln Leu Glu Leu Thr

1 5

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

Arg Ala Leu Thr Val Ala Glu Leu Arg Gly Ser Gly Asp Leu Gln Glu
1 5 10 15

Tyr Leu

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

Thr His Leu Val Ala Ile Gln Asn Lys Glu Glu Ile Glu Tyr Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

Ala Ala Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

Tyr Leu Glu Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Leu Gln Asp Gln Ala Arg Leu Asn Ser Trp Gly Cys Ala Phe Arg Gln
1 5 10 15

Val Cys His Thr
20

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

Trp Gly Cys Ser Gly Lys Leu Ile Cys Thr Thr Ala Val Pro Trp Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

Leu Gly Ile Trp Gly Cys Ser Gly Lys Leu Ile Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

Leu Gln Thr Met Val Lys Leu Phe Asn Arg Ile Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

Asn Ser Val Asp Asp Ala Leu Ile Asn Ser Thr Lys Ile Tyr Ser Tyr
1 5 10 15

Phe Pro Ser Val
20

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

Gln	Tyr	Ile	Lys	Ala	Asn	Ser	Lys	Phe	Ile	Gly	Ile	Thr	Glu	Leu	Lys
1				5				10						15	

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

Pro	Gly	Ile	Asn	Gly	Lys	Ala	Ile	His	Leu	Val	Asn	Asn	Glu	Ser	Ser
1				5				10						15	

The following sequence nos. 53-130 are also included in the present invention:

53. VAKLEAKVAKLEAK
54. AVAKLEAKVAKLEAKGKGKY
55. KVAKLEAKVAKLEAKGKGKY
56. AVHKLEHKVAKLEAKGKGKYVQGEESNDK
57. KGKGKGL
58. KGKGKGLYL
59. WNSG
60. YLEN
61. YDQLVTRVVTHEMAHA
62. EAEEAARLQA
63. SGGKGSFDLEDV
64. AELGGQFHHKSENG
65. GMTAEDLQTRYN
66. TEADYAKNRAVLEY
67. CASQRDRFQVHNPHENDA
68. CKSQSGIEKTTRILHHANISESTQQN
69. CQATAKMAEEQLTTLHVRSEQQS
70. QGPGAPQGPGAPQGPGAPQGPGAP
71. GKGKGKGKGKGG
72. AIHKLEHKIAKLEAKGKGKY
73. PYKCPECGKSFSQKSDLVKHQRTHTG
74. KKK
75. KKKK
76. KKKKK
77. KKKKKK
78. KKKKKKK
79. KKKKKKKK
80. LKYGGENKPGG
81. YGGFM
82. YGGFL
83. RGDS
84. RLDS
85. YIGSR
86. GQGGTHGQWNKP
87. GQWNKPSKOKTN

88. GGLGGYMLGSAMSEPLIH
89. GSDYEDRYYRENMHRYPNQVYYRPVDQYSNQNN
90. RESQAYYQRGAS
91. KYMCNSSCM
92. KYICNSSCM
93. QDLTMKYQIF
94. ILTVILGVL
95. SAYGEPRKL
96. SEIWRDIDF
97. YRPRPRRY
98. TFGLQLELT
99. QKRAA
100. QRRAA
101. DMGHGLRLIHYSYDDVNSTEKG
102. ASQKRPSQRSK
103. VHFFKNIVTPRTP
104. NTWTTCQSIAFPSK
105. SKTSASIGSLCADARMYGVL
106. LINVIHAFQYV
107. PGYPIRALVGD
108. VLGGGCALLRCIPACDSTLPANED
109. PALDSLTPANEA
110. IARFKMFPEVKEK
111. SRLSKVAPVIKARMMEYGTT
112. TPQGRPVAEY
113. TPQGRVAAEY
114. TPQGRVDAEY
115. TPQGRVSAEY
116. EFRADHPFLF
117. HGTVIESLESLNNYFNSSGIDVEEKSLFLDIWRNWQKDG
118. AKFEVNNPQVARAAFNELIRVVHQLLPESSLRKRKRSRC
119. YQGVQQKWDATATELNNALQ
120. MTEQQWNFAGIEAAASAIQ
121. LQNRRGLDLLFLKEGGL
122. RGD
123. GACRGDCLGA
124. GRGDSP
125. CRGDCL

126.CRGDCA

127.CVLNGRME

128.NGRAHA

129.DGRAHA

130.ALNGREESP

CLAIMS

1. A non-dendritic peptide carrier coupled through a linker to a solid phase forming a non-dendritic peptide-solid phase complex, the non-dendritic peptide carrier comprising 10-50 amino acids capable of forming a secondary structure in a benign buffer after liberation from the solid phase, the non-dendritic peptide-solid phase complex further comprising an immunogenic substance and/or an immune mediator coupled on the non-dendritic peptide carrier.

2. A non-dendritic peptide carrier as defined in claim 1 further comprising a covalently bound lipidic moiety.

3. A non-dendritic peptide carrier according to claim 1 or 2 comprising 10-26 amino acids capable of forming a secondary structure in a benign buffer after liberation from the solid phase.

4. A non-dendritic peptide carrier according to any of the preceding claims, wherein the secondary structure is selected from the group α-helices, β-strand, β-turns, γ-turns, zinc-finger structures and combinations thereof.

5. A non-dendritic peptide carrier according to claim 4, wherein the secondary structure is an α-helix.

6. A non-dendritic peptide carrier according to any of the preceding claims, wherein the secondary structure is a result of the inclusion in the peptide of one or more α-helical-, β-strand-, turn- or zinc-finger-inducing sequences of amino acids, respectively, or combinations thereof.

7. A non-dendritic peptide carrier according to any of the preceding claims, wherein the non-dendritic peptide carrier is linked by its C-terminus to a dibranching molecule presenting two molecules of the peptide in parallel.

8. A non-dendritic peptide carrier according to any of the preceding claims wherein the peptide exists as a monomer in a benign aqueous solution.

9. A non-dendritic peptide carrier according to any of the preceding claims and which forms an amphipathic helix in a benign aqueous solution, giving rise to an intramolecular anti-parallel arrangement of two α-helices joined by a turn in a monomer supersecondary hairpin structure.

10. A non-dendritic peptide carrier according to any of claims 1-5 existing as a dimer in a benign aqueous solution.

11. A non-dendritic peptide carrier according to claim 10 and forming amphipathic α-helices in aqueous solutions, giving rise to parallel homodimers of the α-helix coiled-coil type.

12. A non-dendritic peptide carrier according to any of the preceding claims, which carries at least 2 attachment points being derivatizable and accessible functional groups.

13. A non-dendritic peptide carrier according to claim 12 in which the attachment points are chosen from the group of ε-amino groups or derivatized ε-amino groups in the side-chain of lysine or lysine analogous, such as ornithine, α,γ-diaminobutyric acid or α,β-diaminopropinyl acid and a free α-amino group.

14. A non-dendritic peptide carrier according to claim 13 further comprising a subclass of ε-amino groups in the side-chain of other lysine residues and a free α-amino group, said subclass comprising at least one such amino group being protected by a protecting group cleavable by a chemical treatment orthogonal to both Fmoc- and Boc-cleaving chemical treatments.

15. A non-dendritic peptide carrier according to claim 14 comprising as attachment points, a double-functional attachment point, and/or a multiple-functional attachment point.

16. A non-dendritic peptide carrier according to claim 15 in which one of the functional groups of the double-functional attachment points or at least one of the functional groups of the multiple-functional attachment points is reversibly blocked.

17. A non-dendritic peptide carrier according to any of the claims 11-15 in which the side-chain functional groups serving as attachment points are derivatized with secondary structure supporting and derivatizable building blocks.

18. A non-dendritic peptide carrier according to any of the claims 11-15 in which the side-chain functional groups serving as attachment points are derivatized with a dibranching residue being selected from the structures shown in Fig. 4A and Fig. 4B.

19. A non-dendritic peptide carrier according to any of the preceding claims which comprises at least two free carboxylic acids or amino groups located in a side-chain attached near to the C-terminal amino acid.

20. A non-dendritic peptide carrier according to any of the preceding claims, comprising as a lipidic moiety at least one alkyl- and/or alkenyl chain, preferably in the form of a fatty acid bound covalently to the N-terminus of the peptide or to an amino acid side-chain.

21. A non-dendritic peptide carrier according to claim 20 wherein the carbon chain of the alkyl- or alkenyl chain comprises about 4-25 carbon atoms such as 6-20 carbon atoms, preferably 7-17 carbon atoms in length.

22. A non-dendritic peptide carrier according to claim 21 wherein the carbon chain of the lipidic moiety comprises palmitic acid or myristic acid or mixtures thereof.

23. A non-dendritic peptide carrier according to any of the claims 20-22 in which at least one lipidic moiety is bound as a thioester to the peptide, preferably through a cysteine side chain thiol group.

24. A non-dendritic peptide carrier according to any of the claims 20-23 in which the lipidic moiety is the immunostimulatory $palm_3$-Cys-molecule shown in Fig. 4C.

25. A non-dendritic peptide carrier according to any of the claims 20-24 in which the lipidic moiety is coupled to the side chains of amino acids selected from lysine and serine being of alternating chirality, a D-amino acid being followed by a L-amino acid and a L-amino acid being followed by a D-amino acid.

26. A non-dendritic peptide carrier according to any of the preceding claims comprising 15-50 amino acids and comprising in its sequence repeated "heptads" having the form "abcdefg".

27. A non-dendritic peptide carrier according to claim 26 wherein the repeated "heptads" are located in its inner sequence, that is the sequence located within the segment of the peptide carrier corresponding to the segment from amino acid 2-5 to n-(2-5), preferably to the segment from amino acid 3 to n-3, where n is the number of amino acids in the peptide.

28. A non-dendritic peptide carrier according to any of claims 26-27 in which lysine residues occupying "e" and "g" positions are orthogonally side-chain protected compared to lysine residues occupying "b", "c" and "f" positions.

29. A non-dendritic peptide carrier according to any of claims 26-28, in which at least one "f" position is occupied by C.

30. A non-dendritic peptide carrier according to any of the preceding claims which comprises one or more peptide moieties selected from

i) $[TKPR]_N$, in which N is preferably from 1-5 (tuftsin oligomer), muramyldipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine) or variants thereof;

ii) a T-cell stimulatory peptide selected from QYIKANSKFIGITE (tetanus toxoid 830-843) and FNNFTVSFWLHRVKVSASHLE (tetanus toxoid 947-967), DQVHFQPLPPAVVKLSDALI (Mycobacterium tuberculosis 38 kD antigen 350-369), DIEKKIAKMEKASSVFNVVNS (Plasmodium falciparum circumsporozoite protein 378-398), KLLSLIKGVIVHRLEGVE, measles virus F-protein 286-302, LDNIKGNVGKMEDYIKKNNK (Plasmodium falciparum MSP-1, 260-279), LQTMVKLFNRIK, NSVDDALINSTKIYSYFPSV, QYIKANSKFIGITELK, and PGINGKAIHLVNNESS;

iii) a T-cell stimulatory peptide selected from poly T-cell-epitope constructs wherein the T-cell-epitope elements are arranged in a substantially linear construction comprising interposed minimal T-cell epitope peptide segments, preferably without flanking sequences;

iv) a cytokine derived peptide selected from
IFN-gamma(1-39) HGTVIESLESNNYFNSSGIDVEEKSLFLDIWRNWQKDG,
IFN-gamma(95-133) AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC,
TNF (70-80) PSTHVLITHTI, and
IL-1 beta (163-171) VQGEESNDK

v) combinations, including copies thereof.

31. A non-dendritic peptide carrier according to any of the preceding claims which also comprises at least one PNA moi-

ety, said PNA comprising a monomer sequence binding a specific DNA-molecule by hybridization.

32. A non-dendritic peptide carrier according to claim 1-30 comprising at least one functional DNA-intercalator moiety.

33. A non-dendritic peptide carrier according to claim 1-30 comprising at least one DNA oligonucleotide moiety.

34. A non-dendritic peptide carrier according to claim 33 comprising as the DNA moiety an oligonucleotide comprising the sequence (5')PuPuCpGPyPy(3'), in which Pu is a purine base and Py is a pyrimidine base.

35. A non-dendritic peptide carrier according to claim 1-30 comprising a polycationic moiety, preferably poly-lysine, the number of lysines preferably from about 10 to about 15.

36. A non-dendritic peptide carrier according to any of the preceding claims substituting the solid-phase to from about 0.001 to about 5, preferably such as from about 0.01 to about 1, more preferred from about 0.02 to about 0.08, still more preferred from about 0.04 to about 0.06, most preferred from about 0.05 to about 0.1 mmoles pr. gram of solid phase.

37. A non-dendritic peptide carrier according to any of the preceding claims in which the chemical treatment liberating the peptide from the solid-phase bound linker is orthogonal to the chemical treatment used for the cleavage of protecting groups from the intended attachment points in the side-chains of the peptide.

38. A non-dendritic peptide carrier according to claim 37 wherein the peptide is linked to the solid phase through a linker releasing the peptide on contact with water, preferably a glycolic acid linker of the structure given in Figure 4D or a linker releasing the peptide through diketopiperazine formation.

39. A non-dendritic peptide carrier according to any of the preceding claims comprising 10-26 amino acids and containing a lipidic moiety located at the C-terminus or near the C-terminus being preferably palmitic or myristic acid, said peptide containing no free and accessible side-chain functionalities but an unprotected N-terminus α-amino group.

40. A non-dendritic peptide carrier according to any of the claims 1 or 2 comprising 5-50 amino acids said peptide comprising a covalently bound lipidic moiety, preferably palmitic or myristic acid, located at the N-terminus or near the N-terminus, said peptide being attached to the solid phase by a hydrazine-cleavable linker to form a solid phase complex.

41. A non-dendritic peptide carrier according to claim 30, wherein the solid-phase is further derivatized in a defined ratio with a selectively cleavable linker, said linker being cleavable orthogonally to the non-dendritic peptide-linker and designed as a peptide anchoring moiety.

42. A non-dendritic peptide carrier according to any of the preceding claims further comprising a substance having characteristic and measurable spectral or radioactive properties such as UV-absorbing properties, visibly absorbing or fluorescent properties.

43. A non-dendritic peptide carrier according to any of the preceding claims which is non-cyclic.

44. A method for preparing a non-dendritic peptide carrier comprising an immunogenic substance or immune mediator coupled thereon comprising the steps of

i) synthesizing a non-dendritic peptide carrier as defined in any of the preceding claims by chemical solid-phase synthesis on a linker, and

ii) synthesizing the immunogenic and/or immune mediator substance directly on the non-dendritic peptide carrier, and

iii) cleaving the non-dendritic peptide carrier from the solid phase.

45. The method according to claim 44 wherein step ii) further comprises the covalent attachment of other moieties to the peptide carrier by derivatizable groups thereon.

46. The use of a non-dendritic peptide-solid phase complex as defined in any of claims 1-43 as a scaffold for the production of chemical derivatives, characterised by covalently attaching molecules at attachment points, the molecules being selected from polypeptides, carbohydrates, haptens, glycopeptides, lipopeptides, DNA, RNA, PNA, proteins and glycoproteins and combinations thereof.

47. The use of a non-dendritic peptide-solid phase complex as defined in any of claims 1-46 as a scaffold for the stepwise Fmoc- or Boc-based solid-phase peptide synthesis of peptide moieties with defined sequences on the attachment-points of the solid-phase bound non-dendritic peptide.

48. The use of a non-dendritic peptide carrier as defined in any of claims 1-43 as a scaffold-peptide for the incorporation into a Immunostimulating Complex (Iscom) resulting in a non-dendritic peptide carrier-Iscom complex.

49. The use of a non-dendritic peptide carrier-Iscom complex according to claim 48 for the chemical coupling of antigenic substances in an aqueous solution by methods of conjugating peptides to proteins known in the art.

50. A non-dendritic peptide carrier as described in any of claims 1-43 derivatized with one or more peptides having fibronectin-like, laminin-like or vitronectin-like binding activities.

51. A derivatized non-dendritic peptide carrier according to claim 50 wherein the peptide having fibronectin-like, laminin-like or vitronectin-like binding activities comprises in its sequence RGD, GACRGDCLGA, GRGDSP, CRGDCL, CRGDCA, CVLNGRME, NGRAHA, DGRAHA, ALNGREESP, YIGSR, RLDS, or variants thereof.

52. The use of a derivatized non-dendritic peptide carrier according to any of claim 50-51 for the promotion of cell-attachment to plastic surfaces, comprising coating the plastic surface with the derivatized non-dendritic peptide carrier before attaching the cells.

53. The use of a derivatized non-dendritic peptide carrier according to claim 50-52 for the inhibition of tumor growth and metastasis.

54. The use of a derivatized non-dendritic peptide carrier according to any of claims 50-53 for the promotion of wound healing by coating the peptide onto the damaged tissue.

55. The use of a derivatized non-dendritic peptide carrier as defined in any of claims 1-43 for the selection of specifically binding aptamers.

56. A diagnostic component for detecting a molecule or a substance comprising a derivatized non-dendritic peptide carrier as defined in any of claims 1-43 wherein a diagnostic agent is linked.

57. A diagnostic component according to claim 56, wherein the diagnostic agent is an antigen or an antibody.

58. A diagnostic component according to any of claims 56-57 for detecting a molecule which is an aptamer.

59. A diagnostic component according to any of claims 56-58, in which the diagnostic component comprises at least two different diagnostic agents capable of detecting the same or different molecules.

60. A diagnostic component according to any of claims 56-59, in which the diagnostic agent is a molecule selected from the group consisting of a polypeptide; lipopolypeptide; a glycopolypeptide; a phospholipid: a carbohydrate: a lipopolysaccharide; a nucleotide sequence such as a DNA sequence or a RNA sequence; PNA; or any combinations or modifications thereof.

61. A diagnostic component according to any of claims 56-60, in which the amount of the diagnostic agent is effective to detectably react with said molecule to be detected and to which the diagnostic agent is capable of binding.

62. The use of a diagnostic component according to any of claims 56-61 for detection of a molecule, in which the diagnostic composition is incubated with said molecule for a time sufficient for the diagnostic composition to react with the subject and forming a complex and detecting the presence of bound molecule by subjecting said complex to detecting means.

63. The use according to claim 62, for detection of a molecule derived from or indicative of pregnancy, of a disease, such as an infectious disease, an autoimmune disease, a cancerous disease or any other disease wherein an indicative molecule is known.

64. The use according to any of claims 62-63 for detection of a molecule in a sample derived from tissue or tissue extract, a cell culture or an mammal, including a human being.

65. The use according to claim 64, in which the sample is derived from tissue such as a biopsy, or an extract from tissue, a cell, or a cell culture from said mammal.

66. A method of diagnosing a disease or pregnancy in an mammal including a human being, comprising a diagnostic component according to any of claims 56-61 to the mammal, the diagnostic agent detecting in vivo a molecule derived from or indicative of the disease or pregnancy of said mammal.

67. A method of diagnosing a disease or pregnancy in an mammal including a human being, comprising incubating a diagnostic component according to any of claims 56-61 in a sample obtained from said mammal, the diagnostic agent detecting a molecule derived from or indicative of the disease or pregnancy of said mammal and present in the sample.

68. A method according to claim 66 or 67, for detection of a molecule derived from or indicative of an infectious disease, an autoimmune disease, a cancerous disease or the like.

69. A method according to any of claims 66-67, for detection of a molecule in a sample derived from tissue such as a biopsy, or an extract from tissue, a cell, or a cell culture from said mammal.

70. A method according to claim 69, in which the sample is derived from serum, plasma, whole blood, cerebrospinal fluid, seminal or vaginal fluids, saliva, exudates, urine, faeces, or the like.

71. A diagnostic kit comprising a diagnostic component according to any of claims 55-61.

72. A vaccine component comprising a non-dendritic peptide carrier as defined in any of claims 1-43 to which at least one immunogenic agent or mediator is attached.

73. A vaccine component according to claim 72, in which the immunogenic agent is a polypeptide, a glycopeptide, a lipopeptide, a phospholipid, a polysaccharide, a lipopolysaccar-

ide, a carbohydrate, a nucleotide sequence, PNA or any combination or modifications thereof.

74. A vaccine component according to any of claims 72-73, in which the immunogenic agent or mediator is a mixture of peptides.

75. A vaccine component according to any of claims 72-74 wherein the immunogenic agent or mediator is capable of affecting the immunogenic effect or the reaction of the immune system exposed to the vaccine component.

76. A vaccine component according to claim 76, in which the immune mediator is a tuftsin, a cytokine, an adhesion molecule, or a part or modification thereof.

77. A vaccine component according to any of claims 72-74 in which the vaccine component is attached to a second carrier, such as an Immunostimulating Complex (Iscom), a liposome or an immune microparticle, optionally in combination with a mediator or a part thereof having mediator activity and attached to the second carrier.

78. A vaccine composition comprising at least one vaccine component according to any of claims 72-77.

79. A vaccine composition according to claim 78 comprising an effective amount of the vaccine component to confer increased resistance to one or more infection(s) in the mammal, the composition optionally further comprising a pharmaceutically acceptable carrier, vehicle, enhancer or adjuvant, or mixtures thereof.

80. A method of immunizing an mammal, including a human being against a disease, the method comprising administering to the mammal an immunogenically effective amount of the vaccine composition according to claim 78 or 79.

81. A method of immunizing an mammal, including a human, according to claim 80 comprising administering the vaccine composition orally, nasally, rectally, subcutaneously, intradermally, or intramuscularly, or on any mucosal surface.

82. A therapeutic component comprising a non-dendritic peptide carrier as defined in any of claims 1-43 to which at least one therapeutic or prophylactic agent is attached.

83. A therapeutic component according to claim 82 in which the therapeutic or prophylactic agent is a polypeptide, a glycopeptide, a lipopeptide, a phospholipid, a polysaccharide, a lipopolysaccaride, a carbohydrate, a nucleotide sequence, PNA, or any combination or modifications thereof.

84. A therapeutic component according to any of claims 82-83 further comprising at least one mediator capable of controlling or enhancing the effect of the therapeutic or prophylactic agent linked to the carrier.

85. A therapeutic component according to claim 84, in which the mediator is a tuftsin, an immunomodulator including a cytokine, an adhesion molekule, or a part or modification thereof.

86. A therapeutic component according to any of claims 82-85 in which the therapeutic component is attached to a second carrier, such as an Immunostimulating Complex (Iscom), a liposome or a microparticle, optionally in combination with a mediator or a part thereof having mediator activity and attached to the second carrier.

87. A therapeutic component according to any of claims 82-86 further comprising a targeting molecule capable of binding to a target substance present at a specific location in the mammal thereby directing the therapeutic component to said specific location where the therapeutic component is to exert its effect.

88. A therapeutic component according to claim 87, in which the targeting molecule is an antibody.

89. A therapeutic component according to any of claims 87-88 capable of preventing, including preventing relapse, or treating a disease or capable of preventing or disrupting pregnancy.

90. A pharmaceutical composition comprising a therapeutic component according to any of claims 82-89 optionally together with a pharmaceutically acceptable carrier.

91. A pharmaceutical composition according to claim 90 for treatment or prevention of a disease, such as an infectious disease, a cancerous disease or an autoimmune disease.

92. Use of a therapeutic component according to any of claims 82-89 for the preparation of a pharmaceutical composition.

93. A method of treatment and/or prevention of a disease, comprising administering to a patient in need thereof a therapeutically or prophylactically effective amount of a pharmaceutical composition according to any of claims 90-91.

94. Use of a therapeutic component according to any of claims 82-89 for the preparation of a pharmaceutical composition.

95. A detecting component for detecting a molecule or a substance, the component comprising a non-dendritic peptide carrier as defined in any of claims 1-43 to which a detecting agent is linked.

96. A vaccine component according to any of claims 72-79 in which the immunogenic agent is the peptide aa 152-176 from HIV-1 gp120 having the amino acid sequence: GEIKNCSFNISTSIRG-KVQKEYAFF.

97. A vaccine component according to any of claims 72-79 in which the immunogenic agent or mediator is the peptide from HIV-1 gp41 comprising the following amino acid sequence: LERLLL.

98. A vaccine component according to any of claims 72-79 in which the immunogenic agent is YDQLVTRVVTHEMAHA.

99. A vaccine component according to any of claims 72-79 in which the immunogenic agent is EAEEAARLQA.

100. A vaccine component according to any of claims 72-79 in which the immune mediator is IFN-gamma(1-39) HGTVIESLESNNYFN-SSGIDVEEKSLFLDIWRNWQKDG.

101. A vaccine component according to any of claims 72-79 in which the immune mediator is IFN-gamma(95-133) AKFEVNNPQVQRQ-AFNELIRVVHQLLPESSLRKRKRSRC.

102. A vaccine component according to any of claims 72-79 in which the immune mediator is the TNF bioactive sequence (TNF70-80):
Pro-Ser-Thr-His-Val-Leu-Ile-Thr-His-Thr-Ile.

103. A vaccine component according to any of claims 72-79 in which the immune mediator is the Tuftsin peptide TKPR or oligomers thereof.

104. A vaccine component according to any of claims 72-79 in which the immune mediator is the IL-1 beta bioactive sequence: VQGEESNDK/Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys.

105. A vaccine component according to any of claims 72-79 in which the immunogenic agent is the EBA 175-peptide: TLTKEYED-IVLKSHMNRESDD.

106. A therapeutical component according to any of claims 82-94 in which the therapeutical agent is KNGMLKGDKVS.

107. A therapeutical component according to any of claims 82-94 in which the therapeutical agent is CKNKEKKC.

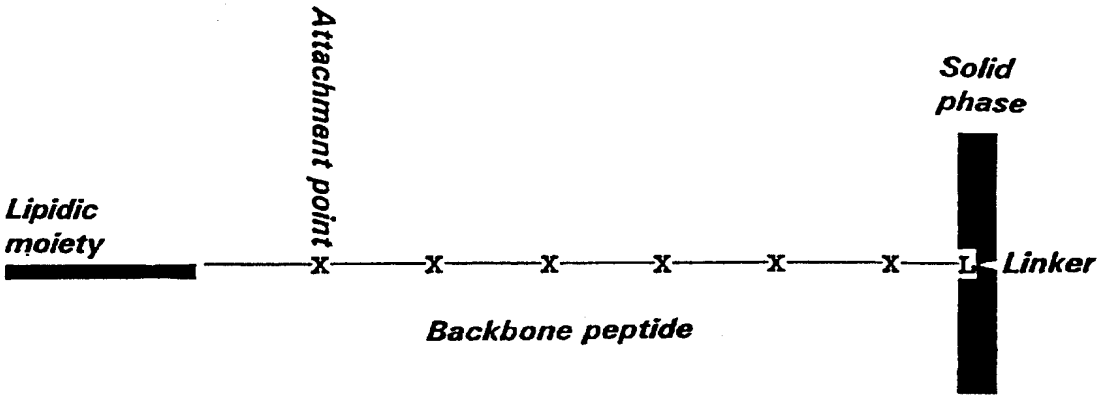

Attachment point
Solid phase
Lipidic moiety
X
X
X
X
X
X
L
Linker
Backbone peptide

Fig. 1A

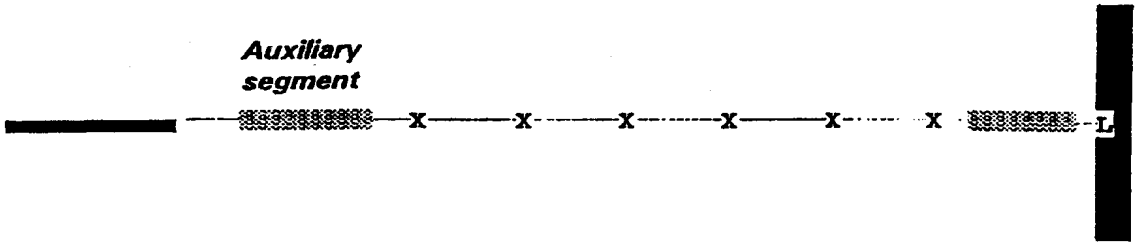

Auxiliary segment
X
X
X
X
X
X
L

Fig. 1B

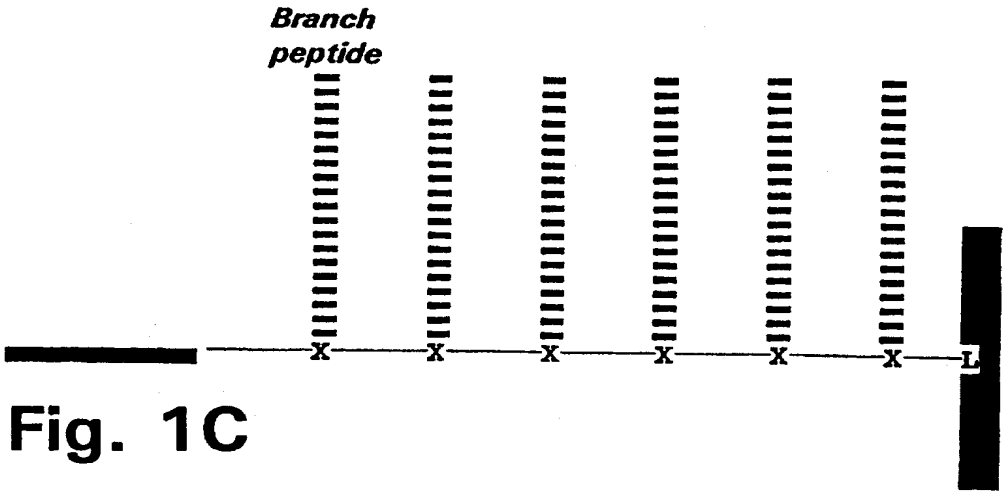


Fig. 1C

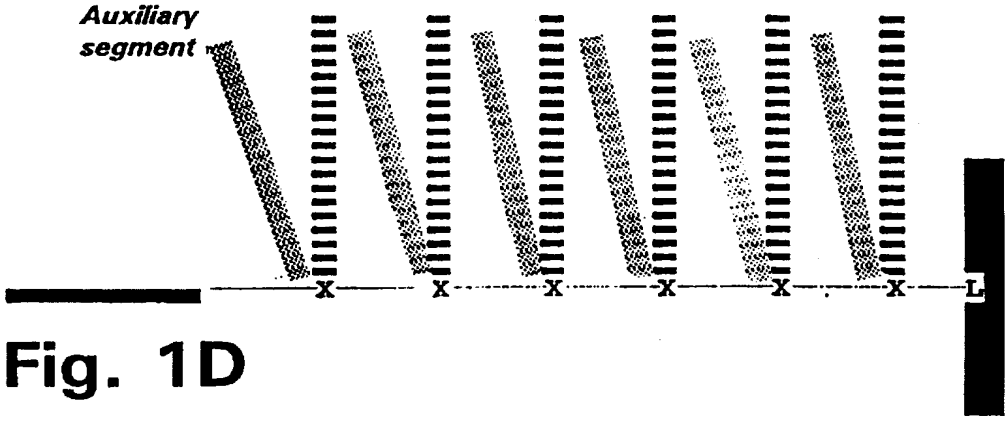


Fig. 1D

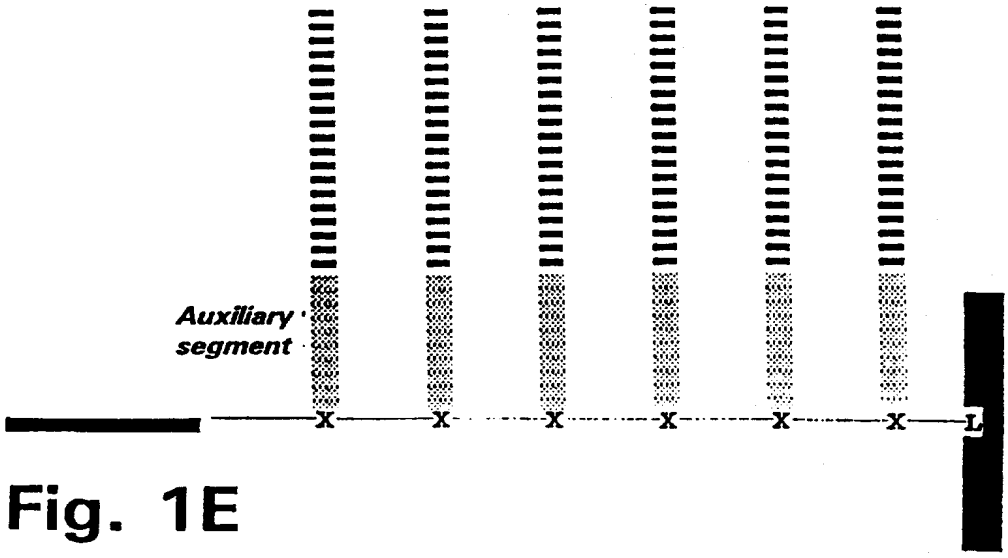
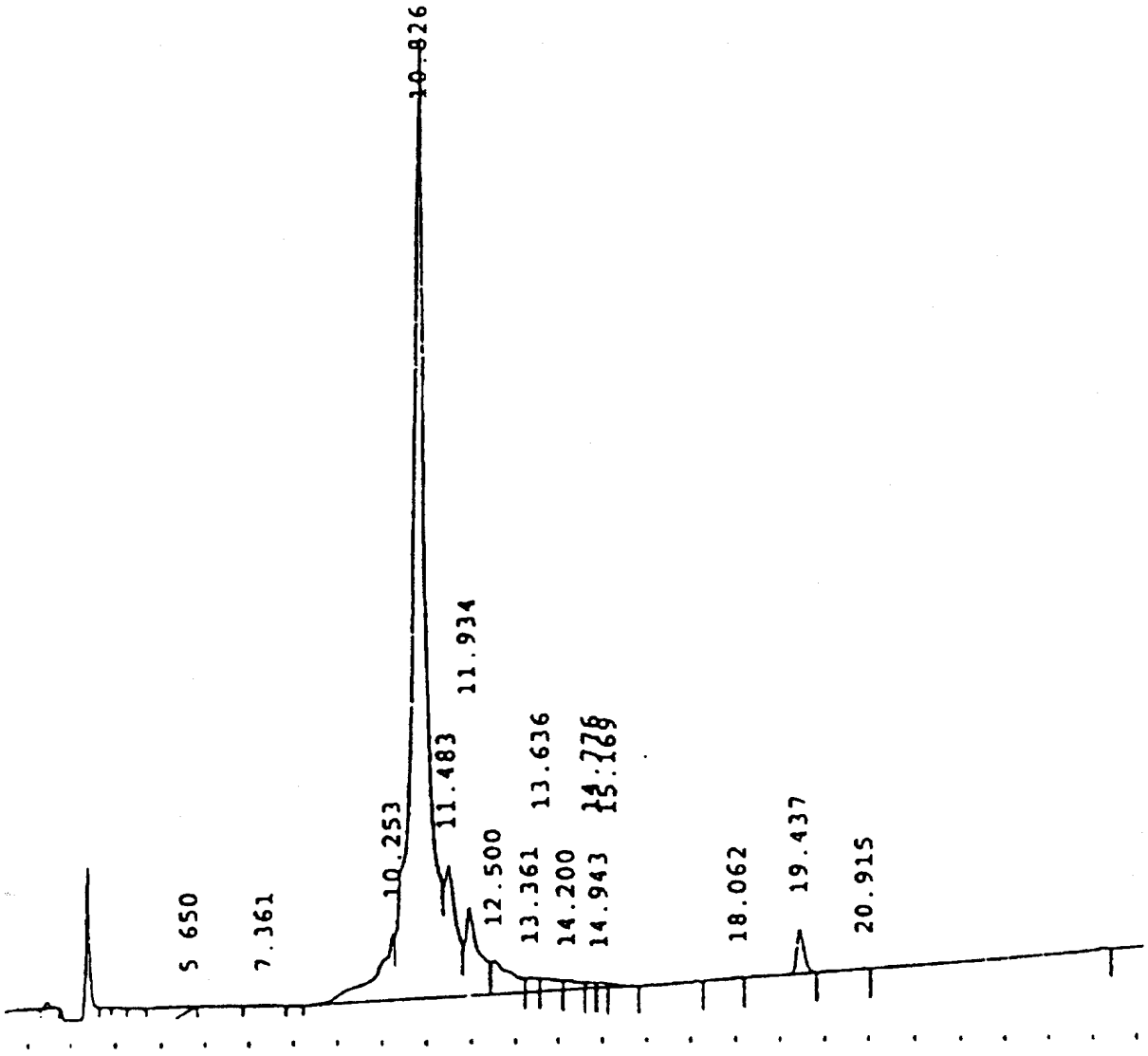


Fig. 1E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11.483
13.636
15.178
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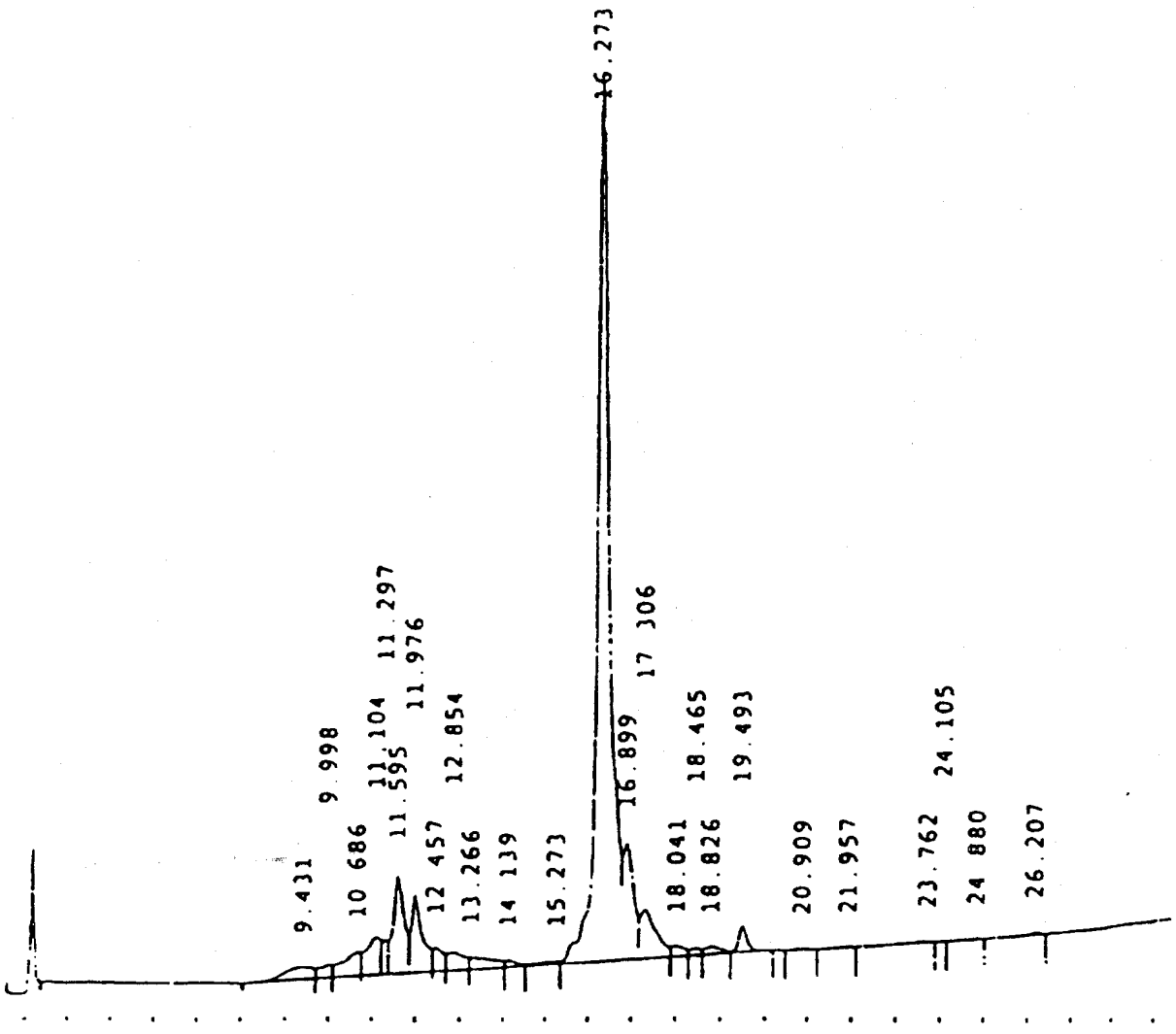
13.361
14.200
14.943
5.650
7.361
18.062
19.437
20.915

Fig. 2A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9.998
10.686
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11.297
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11.976
12.457
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13.266
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16.273
16.899
17.306
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18.826
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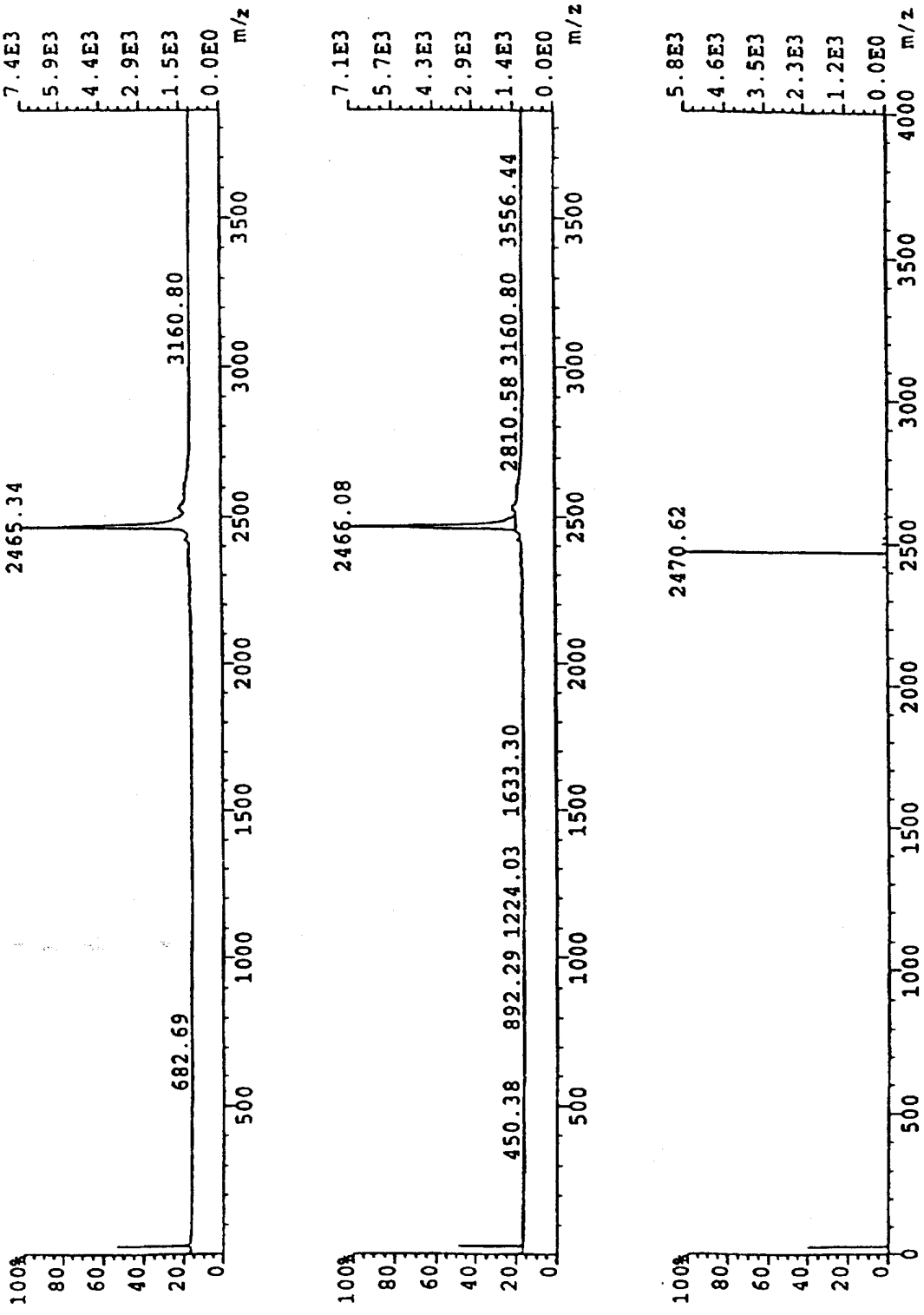
20.909
21.957
23.762
24.105
24.880
26.207

Fig. 2B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m/z
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682.69
7.1E3
5.7E3
4.3E3
2.9E3
1.4E3
0.0E0
2466.08
2810.58
3160.80
3556.44
450.38
892.29
1224.03
1633.30
5.8E3
4.6E3
3.5E3
2.3E3
1.2E3
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100
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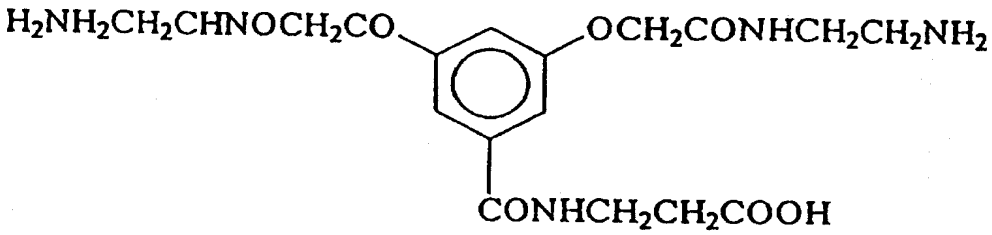
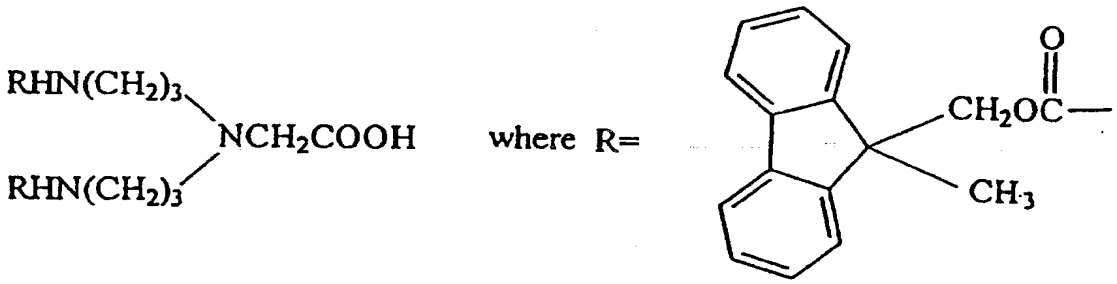
40
20
0
500
1000
1500
2000
2500
3000
3500
4000

Fig. 3



H2Dab-OH

Fig. 4A



Fmoc

Fig. 4B

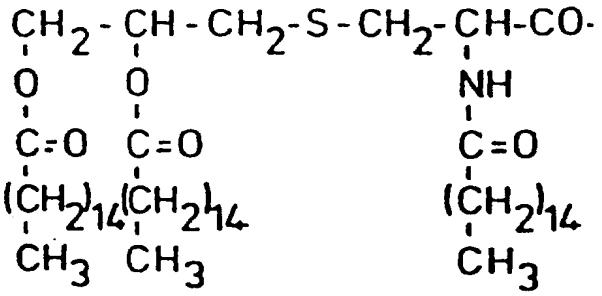
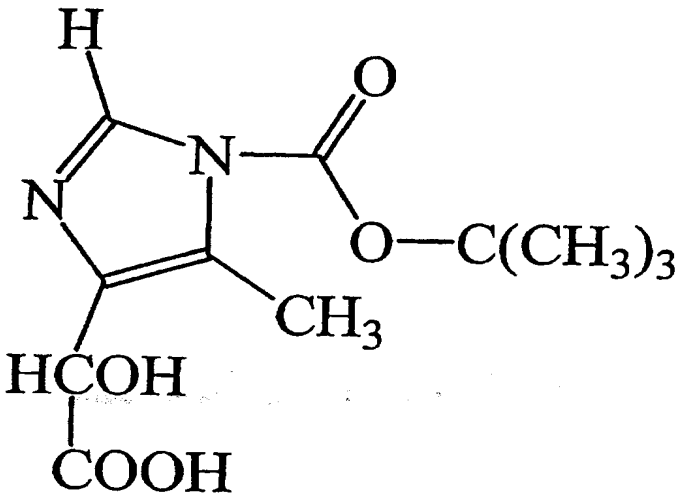
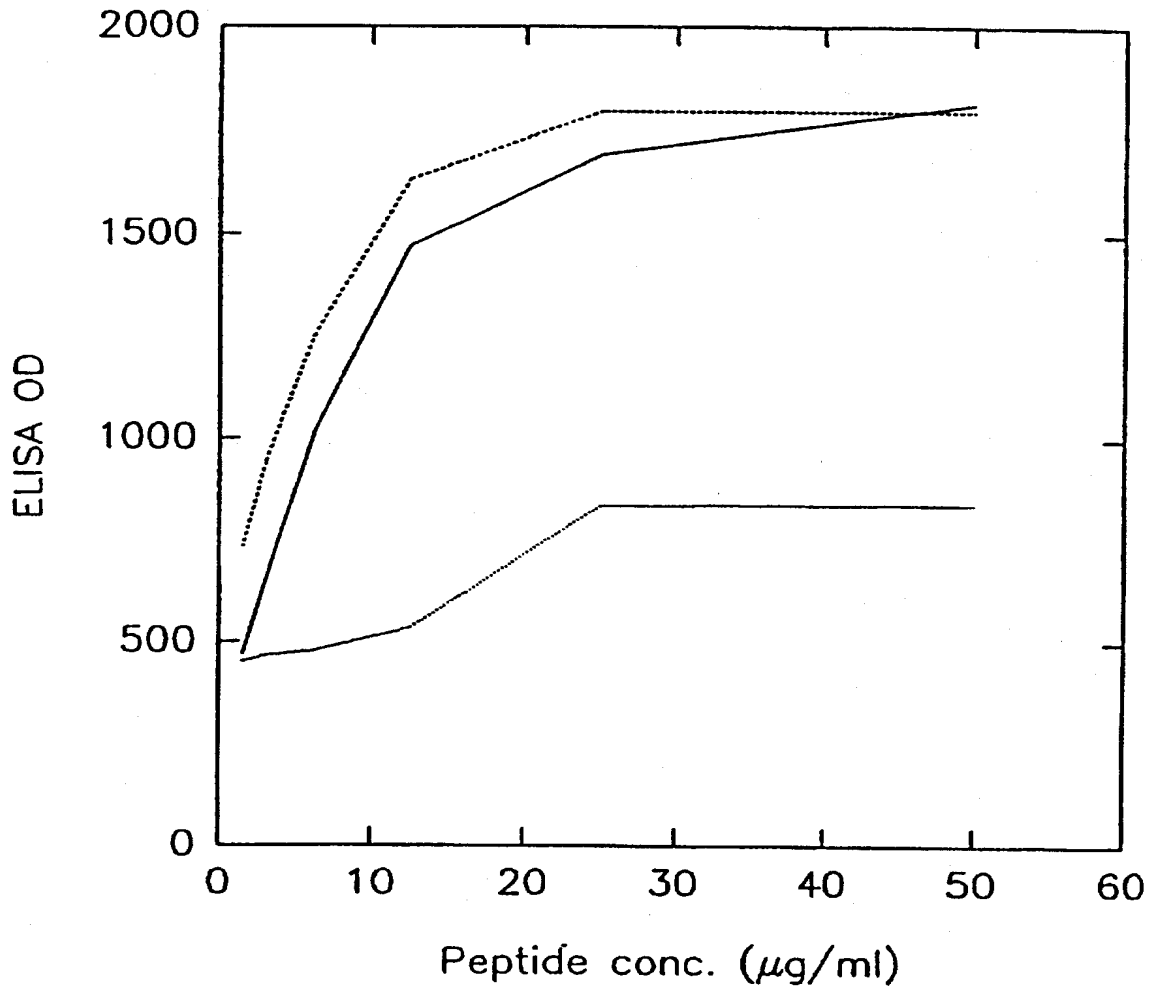


Fig. 4C



2-[1-*tert*-butoxycarbonyl-5-methylimidazol-4-yl]-2-hydroxy acetic acid

Fig. 4D

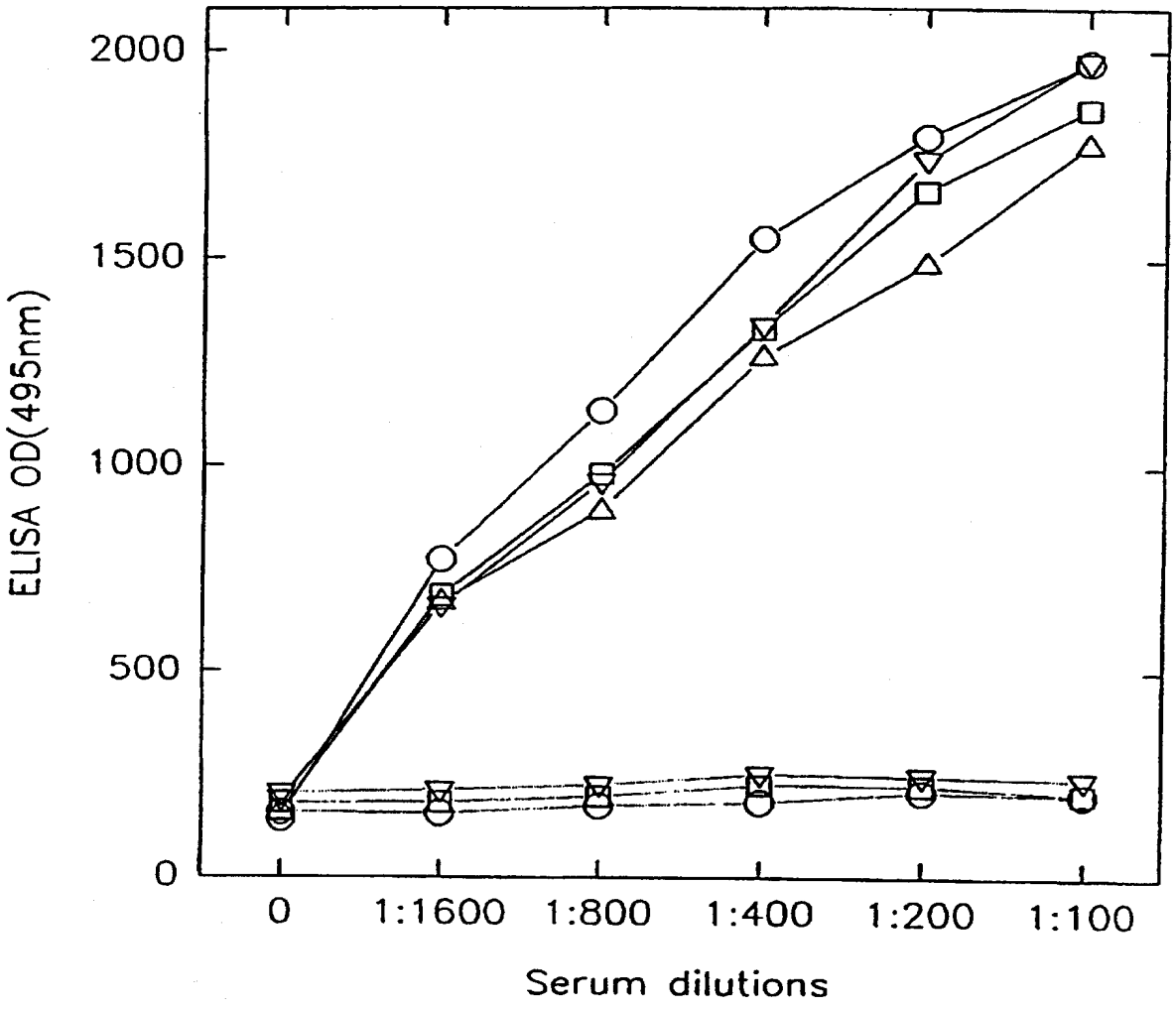


—— = Coating of gp41 (aa 598-609) linked to carrier at pH 2.5

------ = Coating of gp41 (aa 598-609) linked to carrier at pH 9.2

····· = Coating of gp41 (aa 598-609) without carrier at pH 2.5

Fig. 5

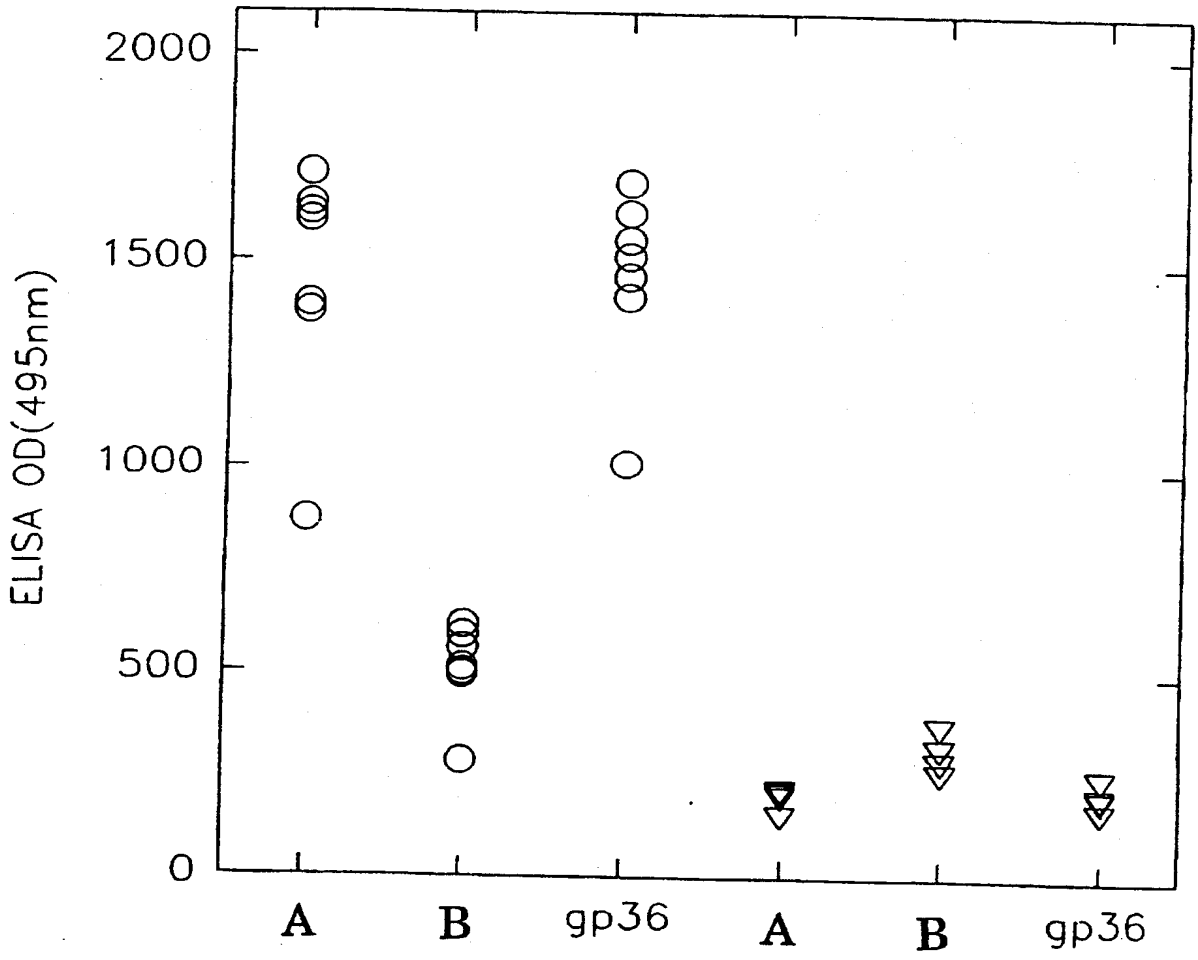


..... = HIV-2 seronegative donors

—— = HIV-2 seropositive donors

Symbols indicating individual donors

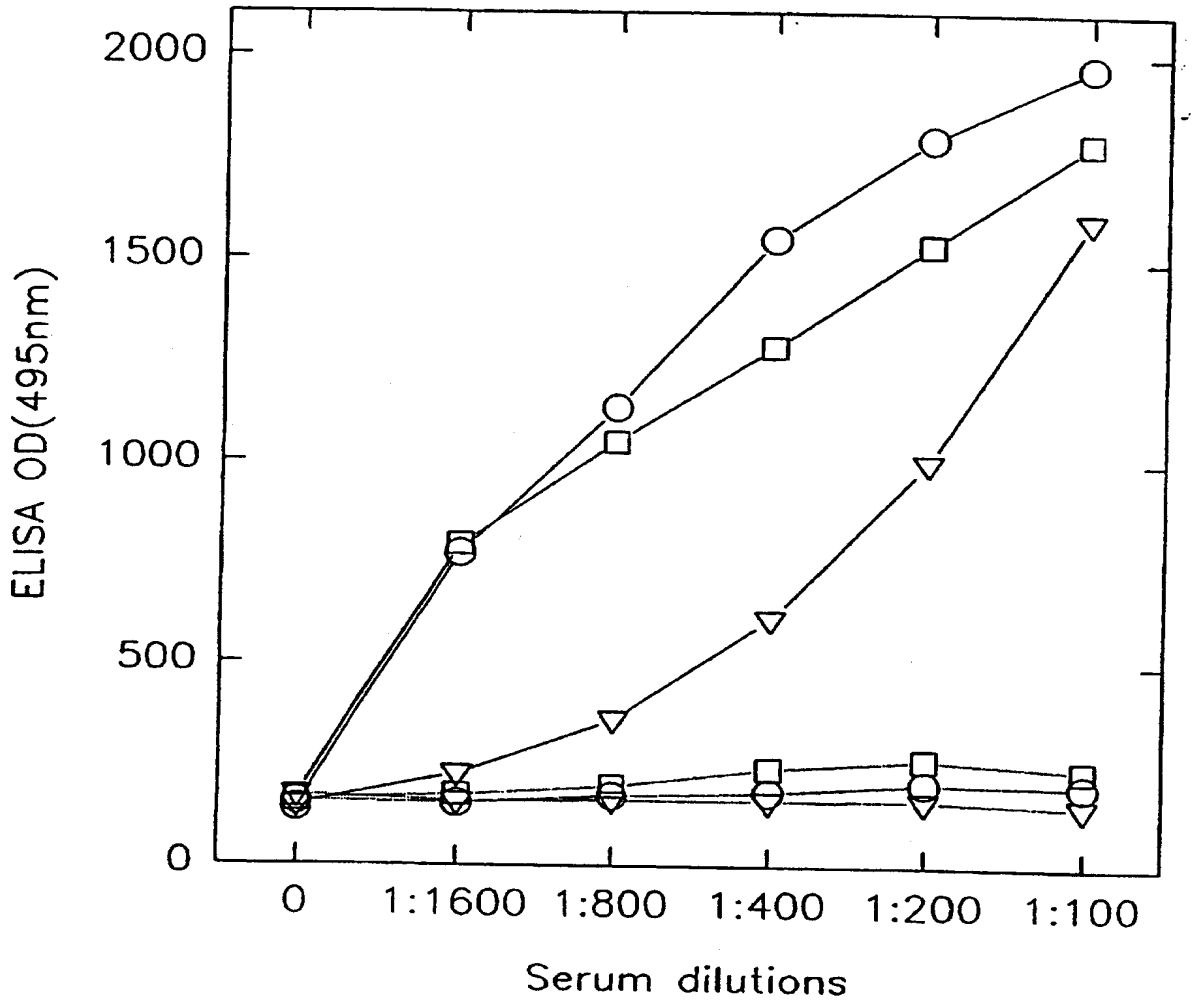
Fig. 6



o = HIV-2 seropositive
▽ = HIV-2 seronegative

A = peptide linked to non-dendritic peptide carrier
B = peptide alone

Fig. 7



— = HIV-2 seropositive donor

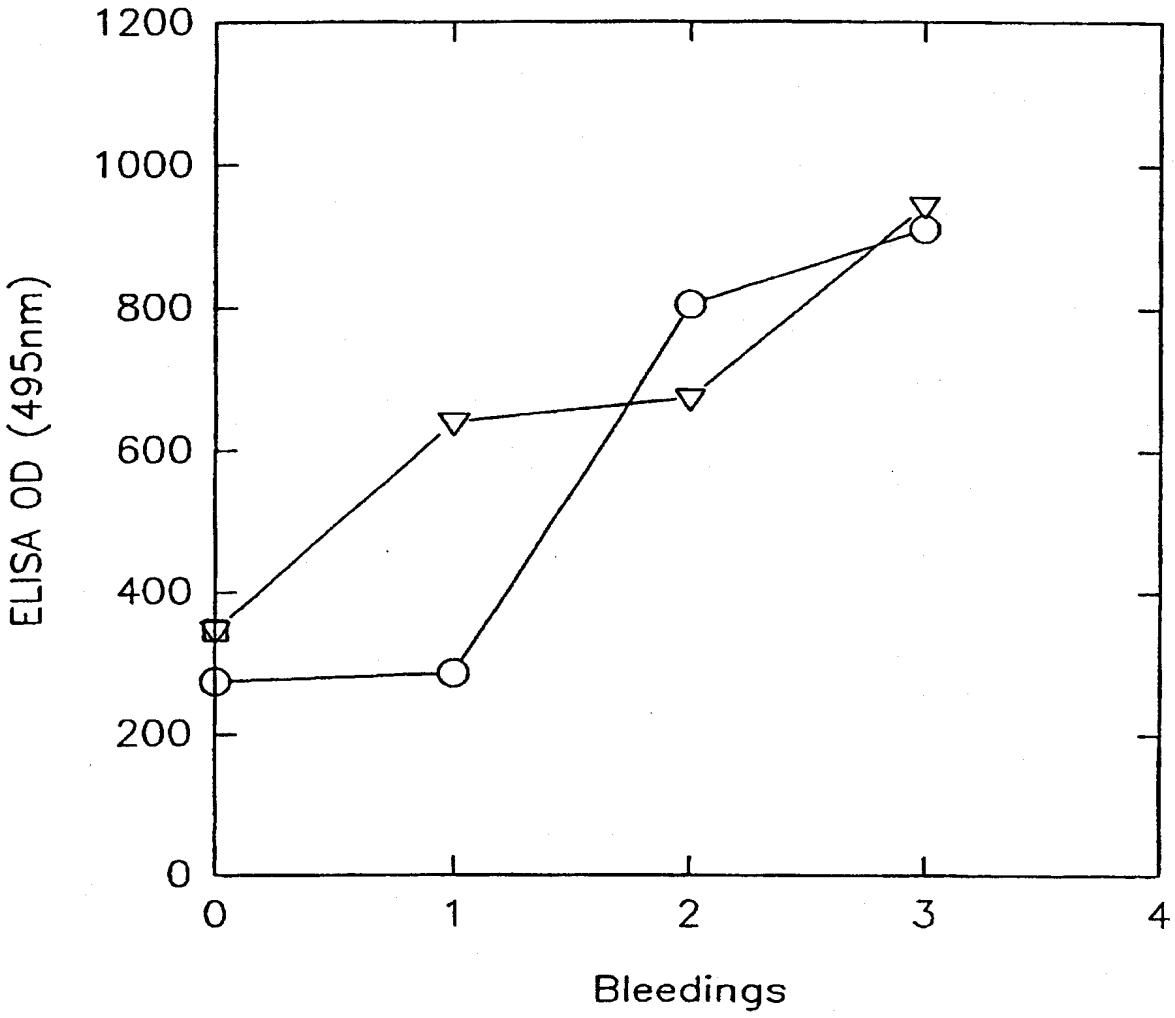
····· = HIV-2 seronegative donor

o = gp36 (aa 587-605) linked to carrier

▽ = gp36 (aa 587-605) not linked to carrier

□ = recombinant gp36

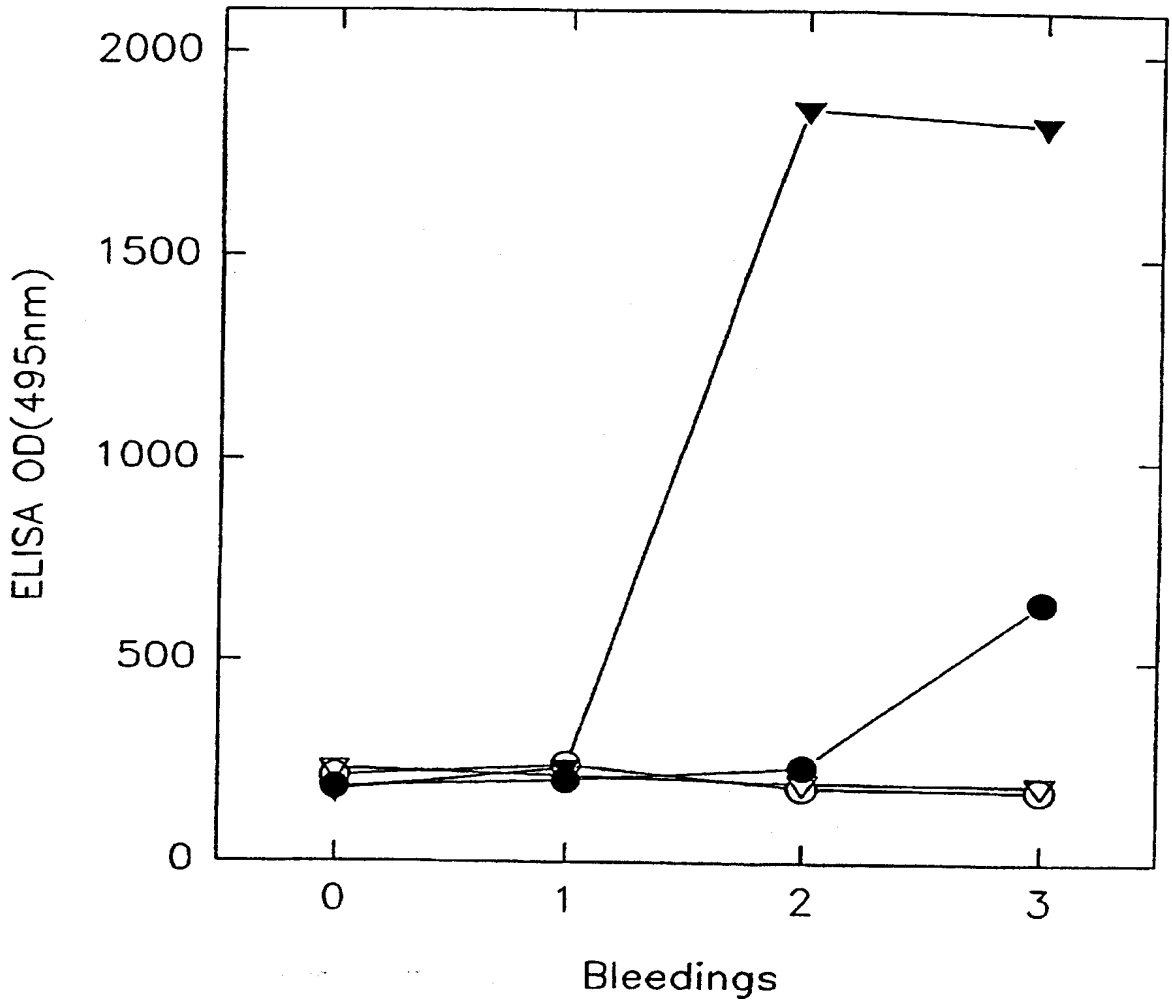
Fig. 8



o = without aluminium hydroxide and with PPD

▽ = with aluminium hydroxide and with PPD

Fig. 9



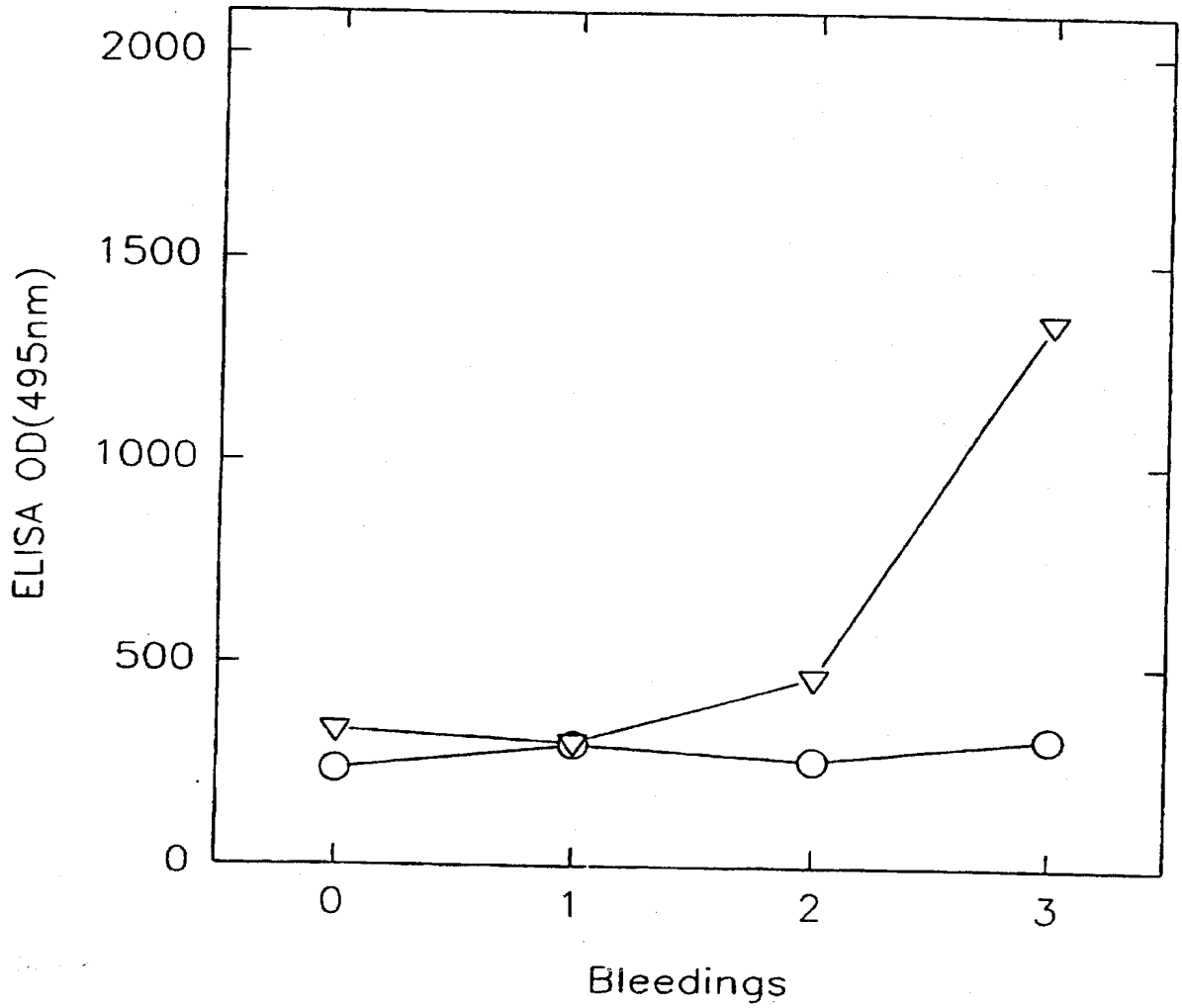
● = Immunization i.p. with erythrocyte binding peptide linked to carrier

o = As ● but without erythrocyte binding peptide

▼ = Immunization s.c. with erythrocyte binding peptide linked to carrier together with Freunds adjuvant

▽ = As ▼ but without erythrocyte binding peptide

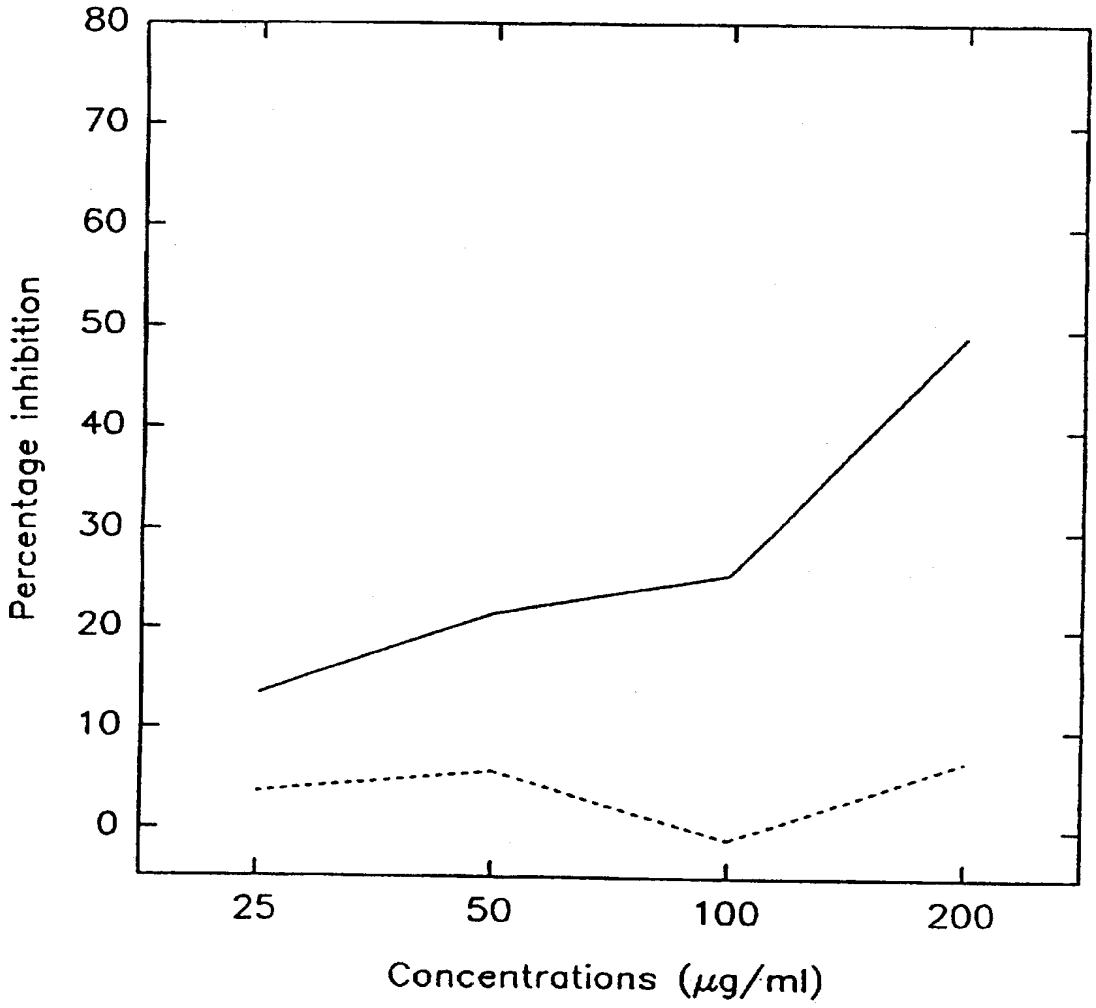
Fig. 10



▽ = Immunization s.c. with gp120 linked to carrier and with Freunds adjuvant

o = Immunizaton i.p. with gp120 linked to carrier

Fig. 11



—— = Inhibition of IL-6 secretion by beta-2-glycoprotein I peptide linked to carrier

---- = as above without being linked to carrier

Fig. 12

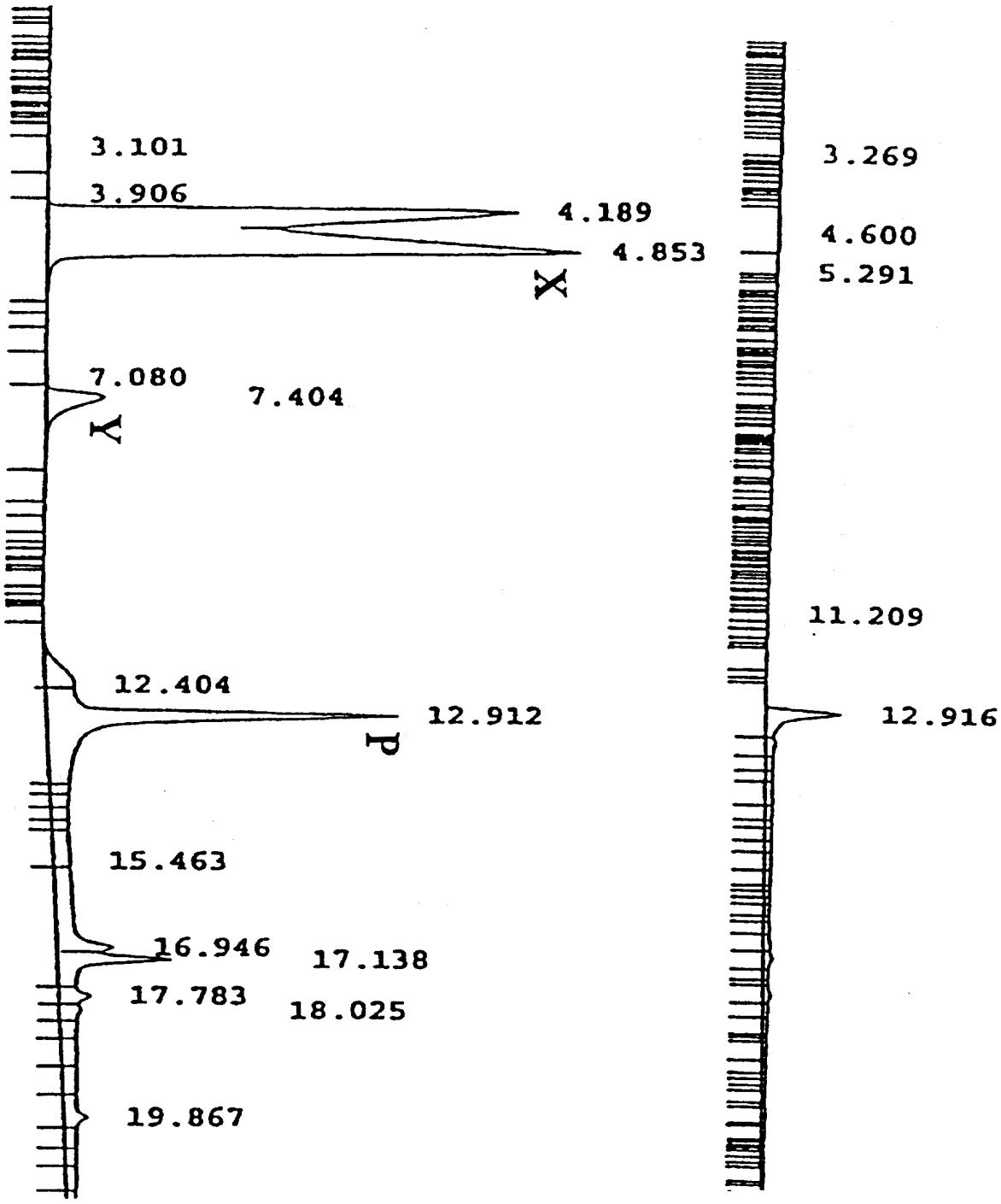
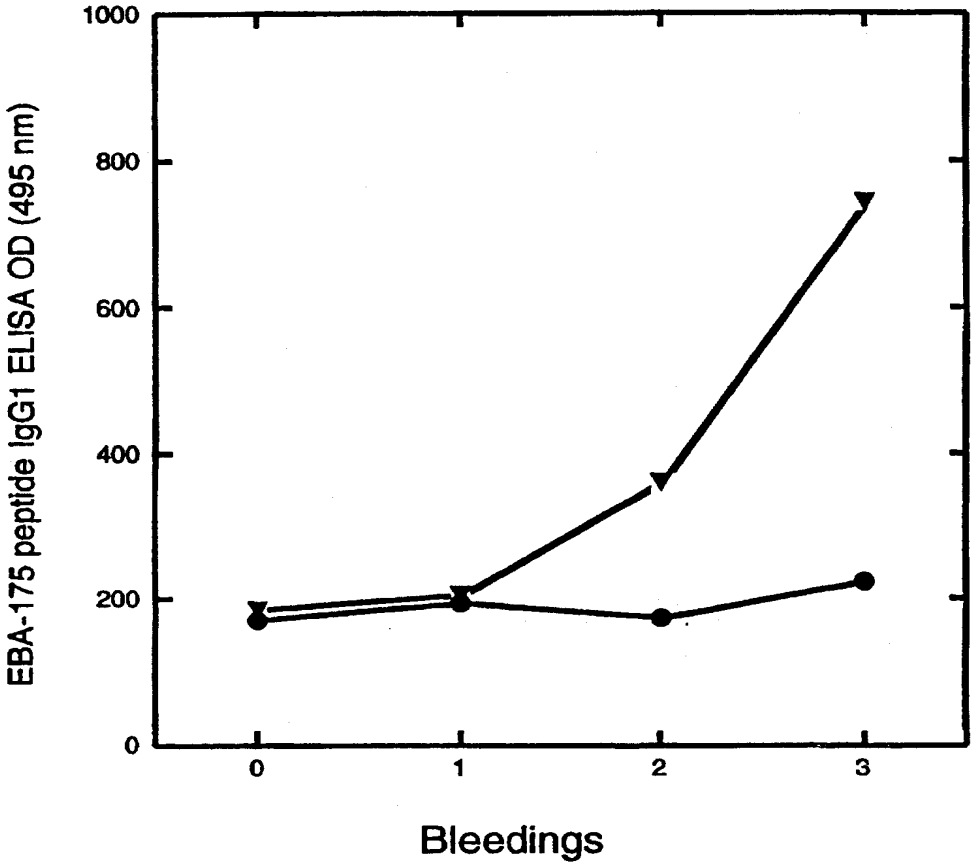

3.101
3.906
4.189
4.853
7.080
7.404
12.404
12.912
15.463
16.946
17.138
17.783
18.025
19.867
3.269
4.600
5.291
11.209
12.916

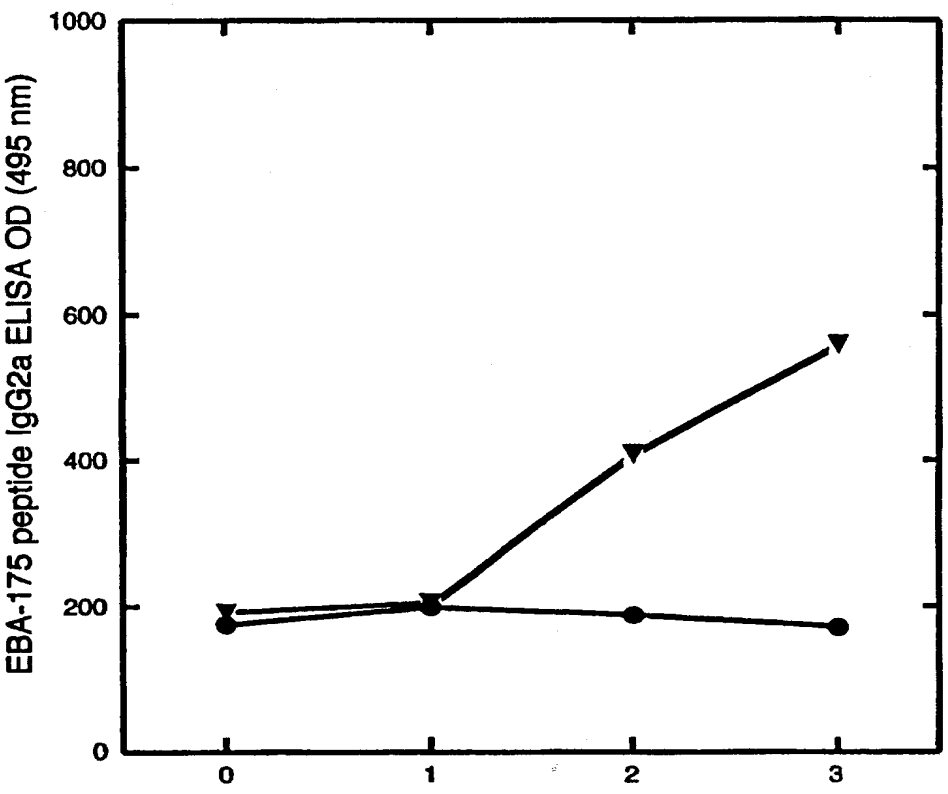
Fig. 13



● Non-dendritic peptide carrier derivatized with EBA-175 peptide i.p.

▼ Non-dendritic peptide carrier derivatized with EBA-175 peptide + Freunds adjuvant s.c.

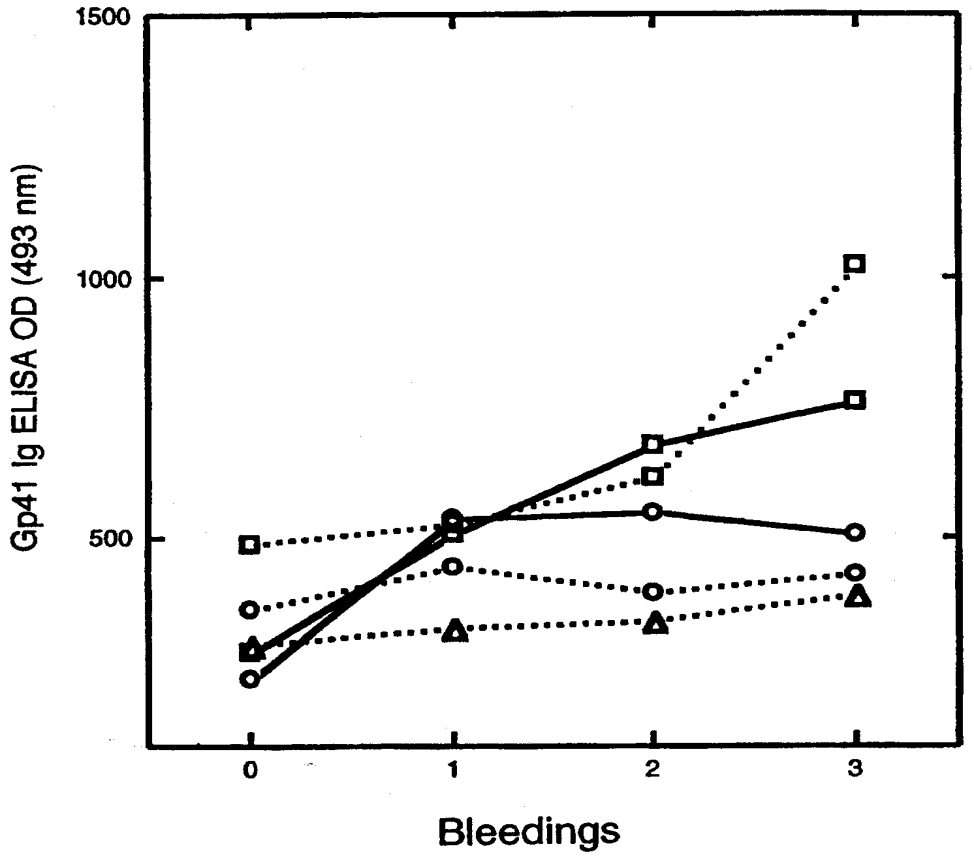
Fig. 14



● Non-dendritic peptide carrier derivatized with EBA-175 peptide i.p.

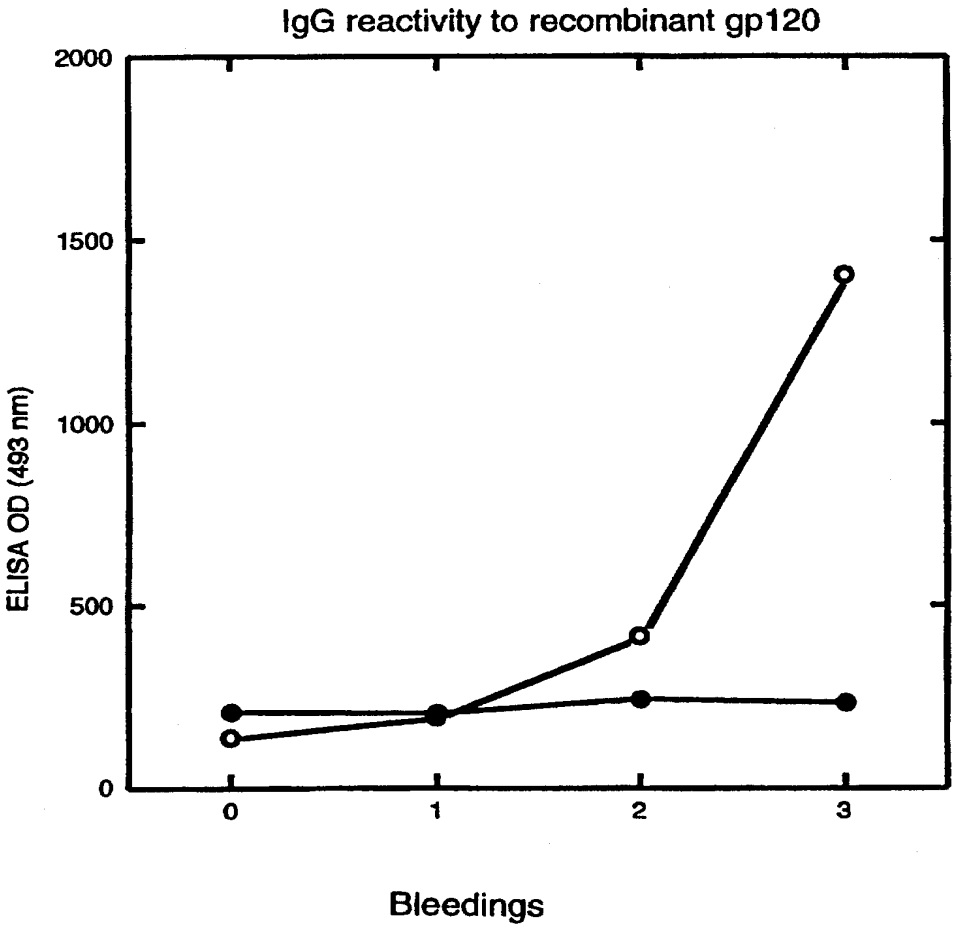
▼ Non-dendritic peptide carrier derivatized with EBA-175 peptide + Freunds adjuvant s.c.

Fig. 15



- Non-dendritic peptide carrier derivatized with gp41 peptide i.p.
- Non-dendritic peptide carrier derivatized with gp41 peptide + Freunds adjuvant
- Non-dendritic peptide carrier derivatized with gp41 peptide + non-dendritic peptide carrier derivatized with gp120 peptide i.p.
- Non-dendritic peptide carrier derivatized with gp41 peptide + non-dendritic peptide carrier derivatized with gp120 peptide + Freunds adjuvant s.c.
- Non-dendritic peptide carrier derivatized with gp120 peptide + Freunds adjuvant s.c.

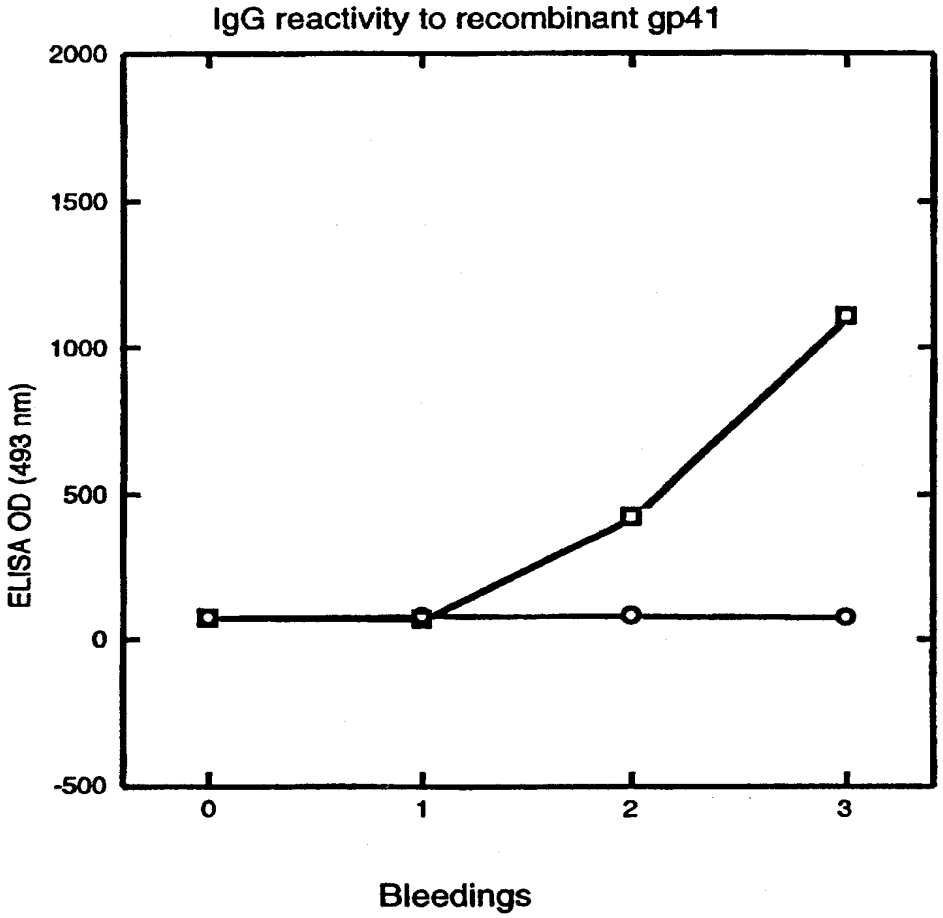
Fig. 16



○ Non-dendritic peptide carrier derivatized with gp120 peptide + Freunds adjuvant s.c.

● Non-dendritic peptide carrier derivatized with gp120 peptide i.p.

Fig. 17



□ Non-dendritic peptide carrier derivatized with gp41 peptide + non-dendritic peptide carrier derivatized with gp120 peptide + Freunds adjuvant s.c.

○ Non-dendritic peptide carrier derivatized with gp41 peptide + Freunds adjuvant s.c.

Fig. 18

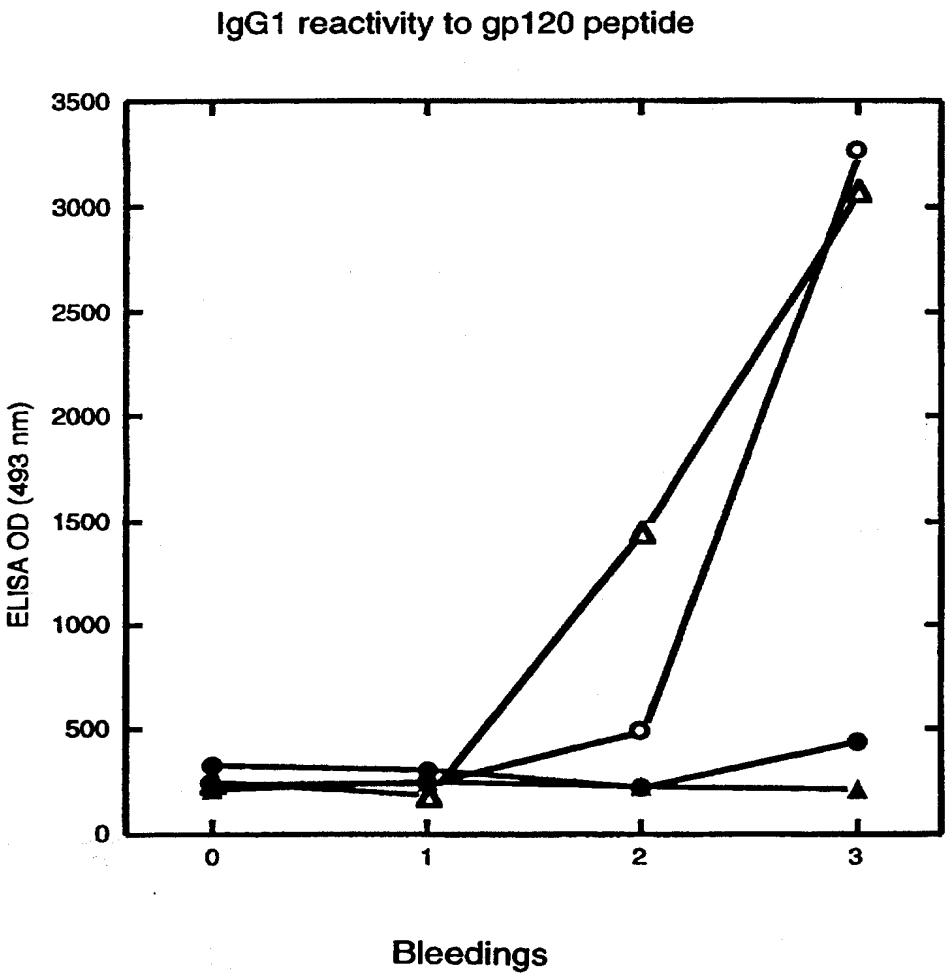

IgG1 reactivity to gp120 peptide
ELISA OD (493 nm)
3500
3000
2500
2000
1500
1000
500
0
0
1
2
3
Bleedings
s.c.
i.p.
Non-dendritic peptide carrier derivatized with gp120 peptide
Non-dendritic peptide carrier derivatized with gp120 peptide + non-dendritic peptide carrier derivatized with gp41 peptide

Fig. 19

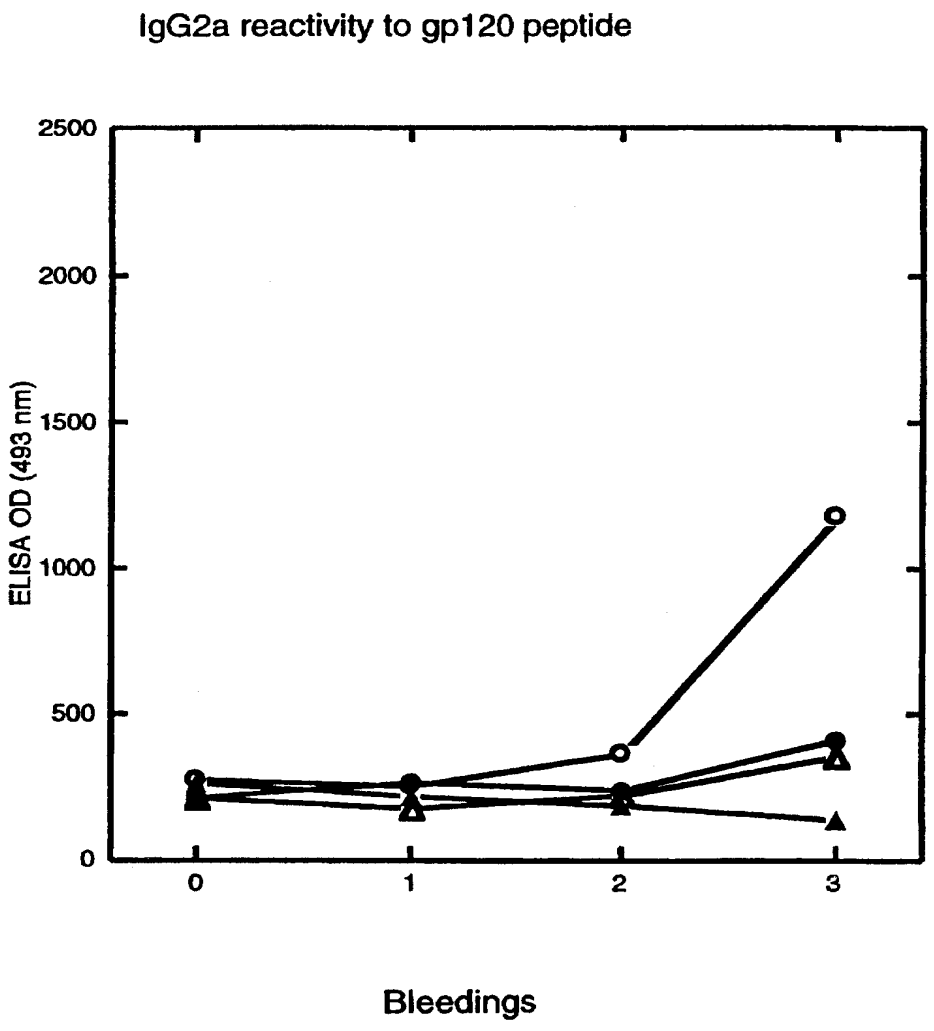

IgG2a reactivity to gp120 peptide
2500
2000
1500
1000
500
0
ELISA OD (493 nm)
0
1
2
3
Bleedings
s.c.
i.p.
Non-dendritic peptide carrier derivatized with gp120 peptide
Non-dendritic peptide carrier derivatized with gp120 peptide +
non-dendritic peptide carrier derivatized with gp41 peptide

Fig. 20

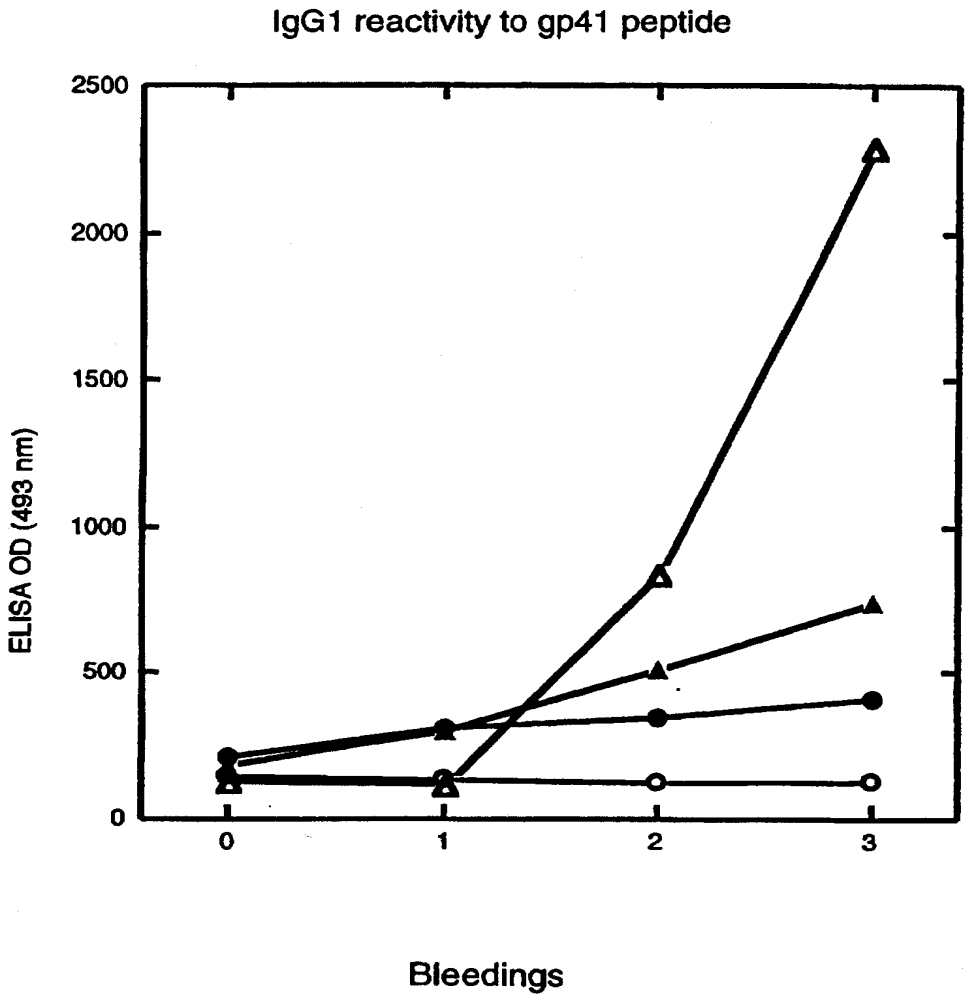

IgG1 reactivity to gp41 peptide
ELISA OD (493 nm)
2500
2000
1500
1000
500
0
0
1
2
3
Bleedings
s.c.
i.p.
Non-dendritic peptide carrier derivatized with gp120 peptide
Non-dendritic peptide carrier derivatized with gp120 peptide +
non-dendritic peptide carrier derivatized with gp41 peptide

Fig. 21

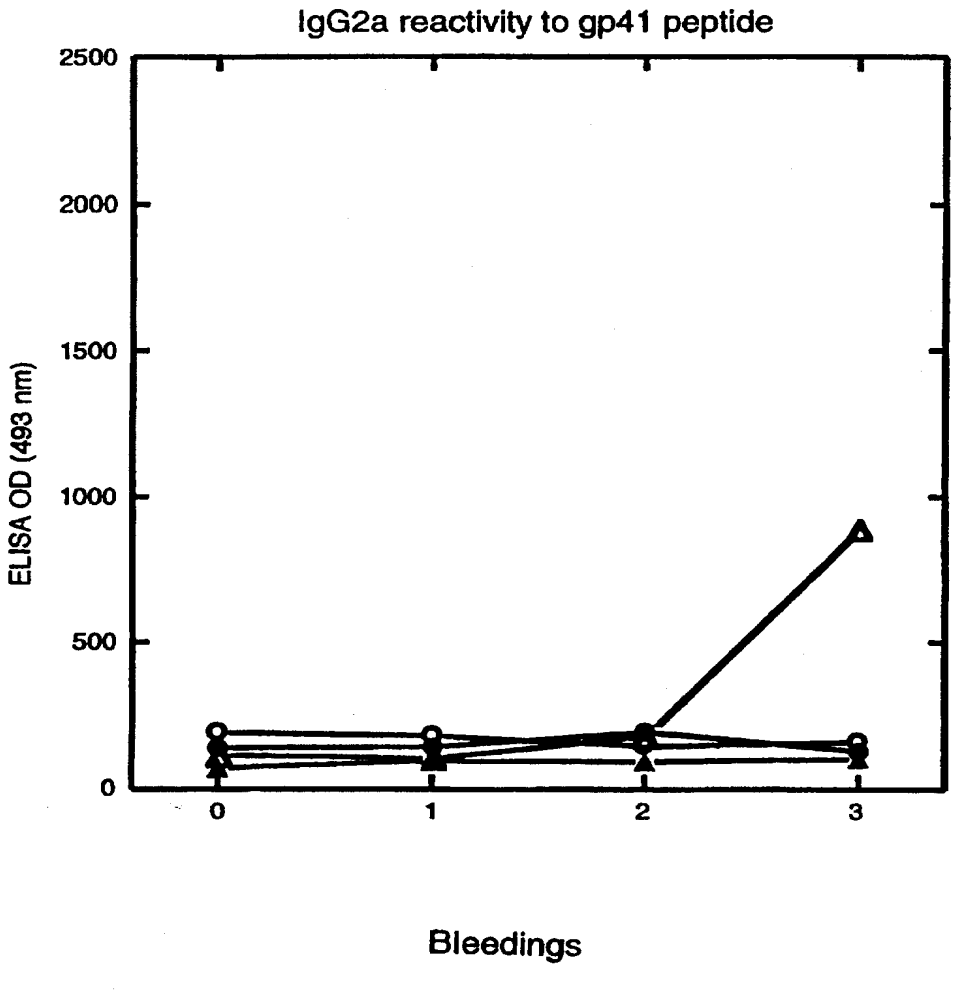
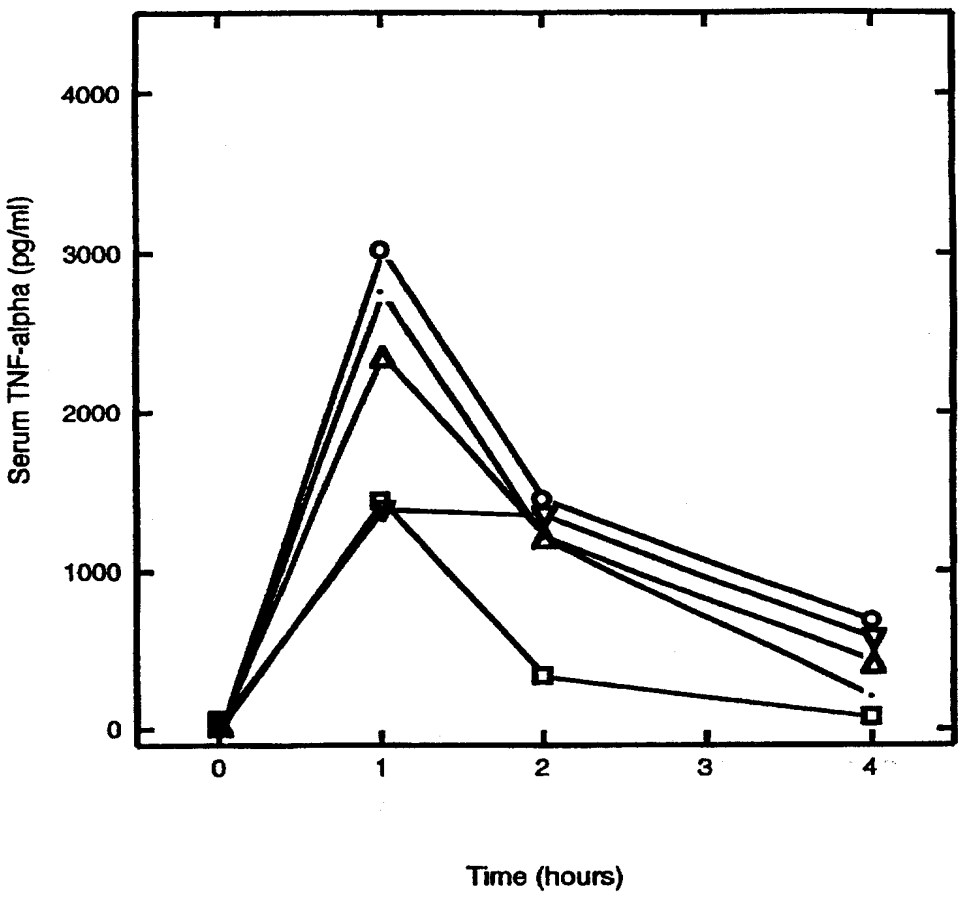
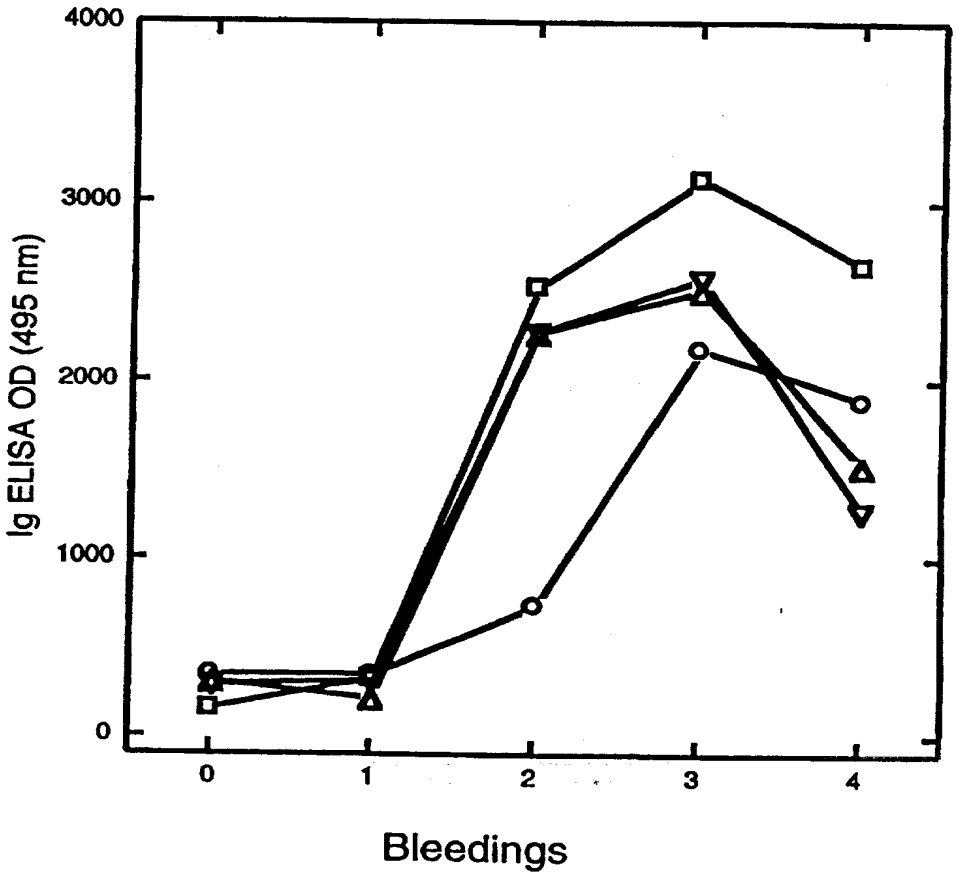

IgG2a reactivity to gp41 peptide
ELISA OD (493 nm)
2500
2000
1500
1000
500
0
0
1
2
3
Bleedings
s.c.
i.p.
Non-dendritic peptide carrier derivatized with gp120 peptide
Non-dendritic peptide carrier derivatized with gp120 peptide + non-dendritic peptide carrier derivatized with gp41 peptide

Fig. 22



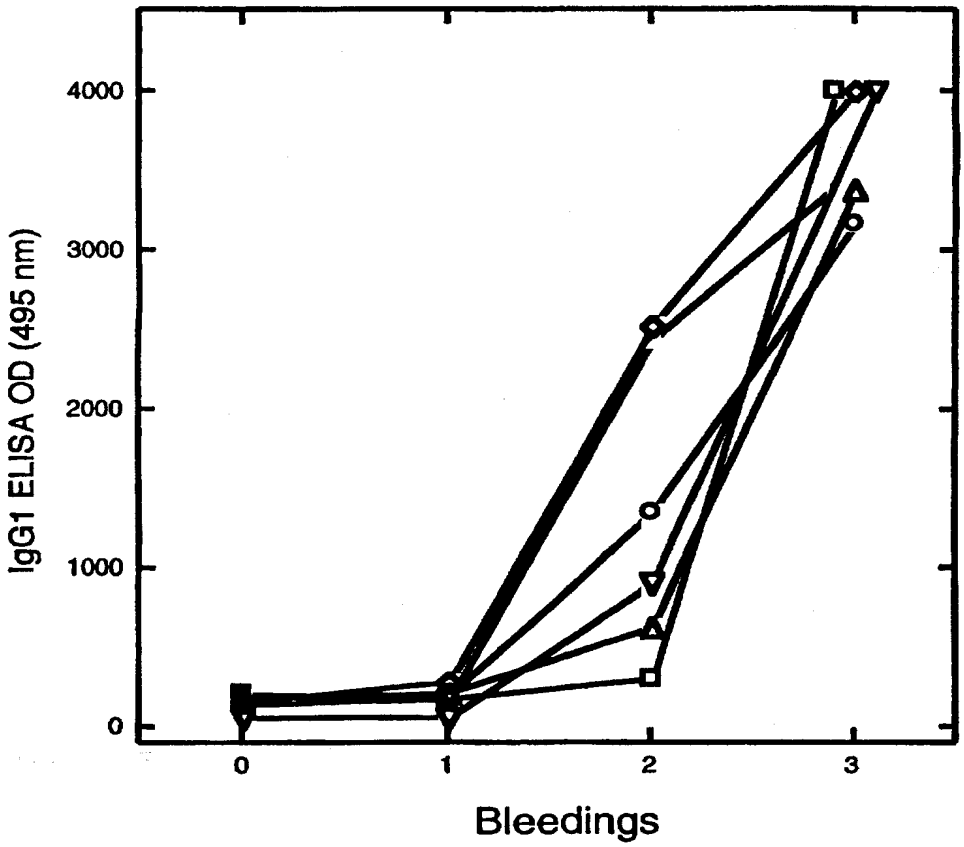
- ○ LPS
- □ Non-dendritic peptide carrier derivatized with CKNKEKKC peptide + LPS
- △ CKNKEKKC peptide + LPS
- ▽ Non-dendritic peptide carrier derivatized with KNGMLKGDKVS peptide + LPS
- • Non-dendritic peptide carrier + LPS

Fig. 23



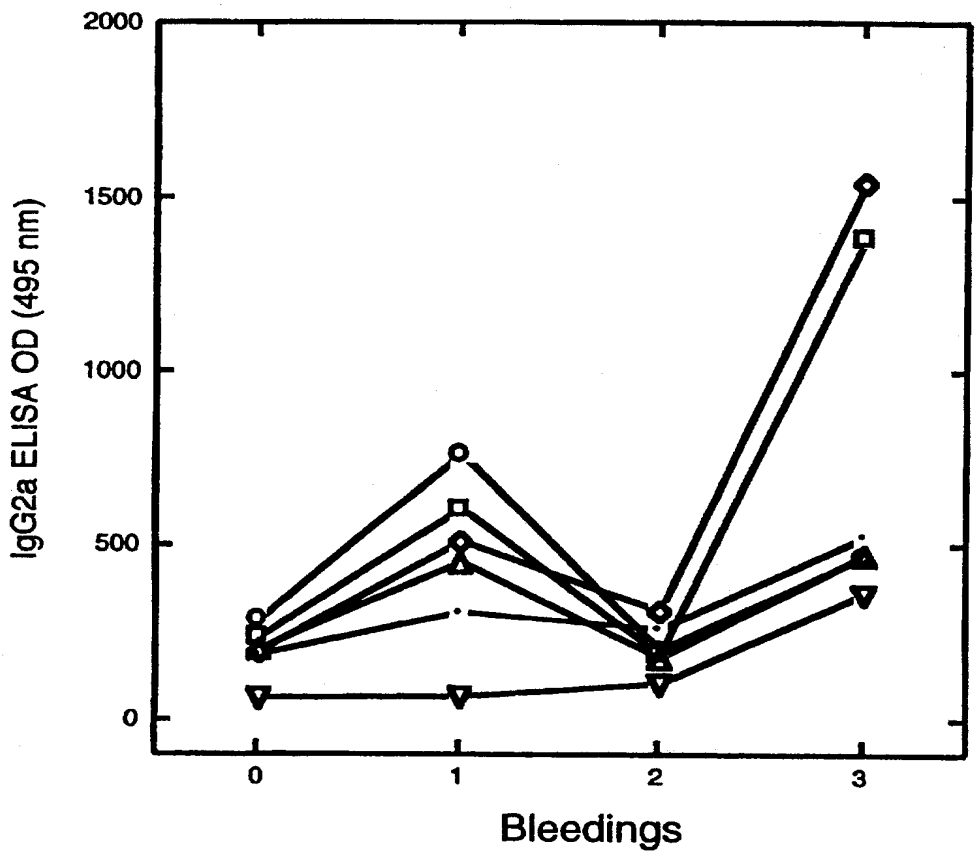
- Non-dendritic peptide carrier derivatized with EBA(1076-96) peptide + Freunds adjuvant
- Non-dendritic peptide carrier derivatized with EBA(1076-96) peptide + Freunds adjuvant + IL10
- Non-dendritic peptide carrier derivatized with EBA(1076-96+1086-1104) peptide + Freunds adjuvant
- Non-dendritic peptide carrier derivatized with EBA(1076-96+1086-1104) peptide + Freunds adjuvant + IL10

Fig. 24



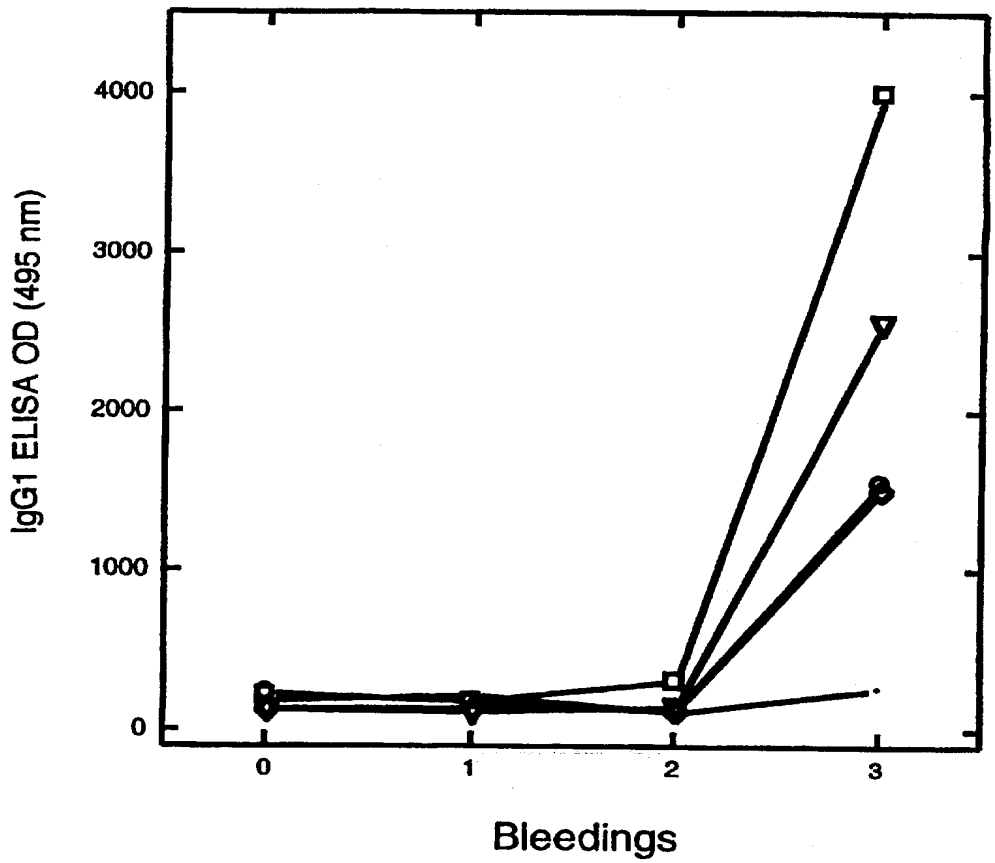
- Non-dendritic peptide carrier derivatized with L1 peptide+alumn s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + Freunds complete adjuvant s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + rek. IFNgamma + alumn s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + rek. IL-12 + alumn s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + rek. TNF + alumn s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + rek. IL-4 + alumn s.c.

Fig. 25



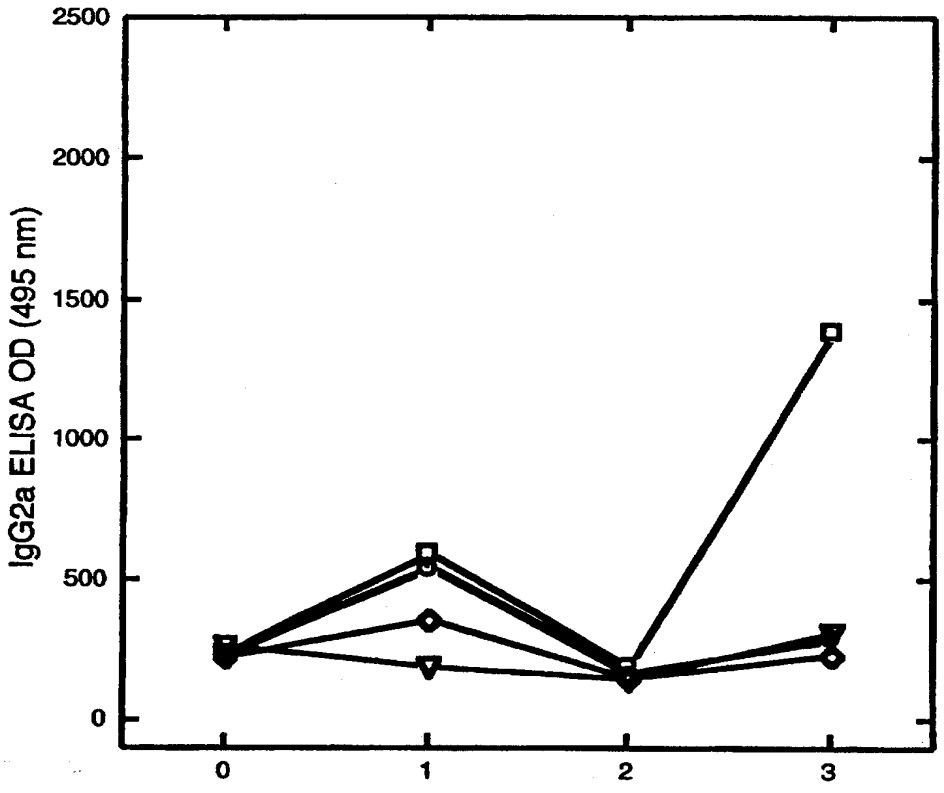
- ○ Non-dendritic peptide carrier derivatized with L1 peptide + alumn s.c.
- □ Non-dendritic peptide carrier derivatized with L1 peptide + Freunds complete adjuvant s.c.
- ▽ Non-dendritic peptide carrier derivatized with L1 peptide + rek. IFNgamma + alumn s.c.
- • Non-dendritic peptide carrier derivatized with L1 peptide + rek. IL-12 + alumn s.c.
- ◇ Non-dendritic peptide carrier derivatized with L1 peptide + rek. TNF + alumn s.c.
- △ Non-dendritic peptide carrier derivatized with L1 peptide + rek. IL-4 + alumn s.c.

Fig. 26



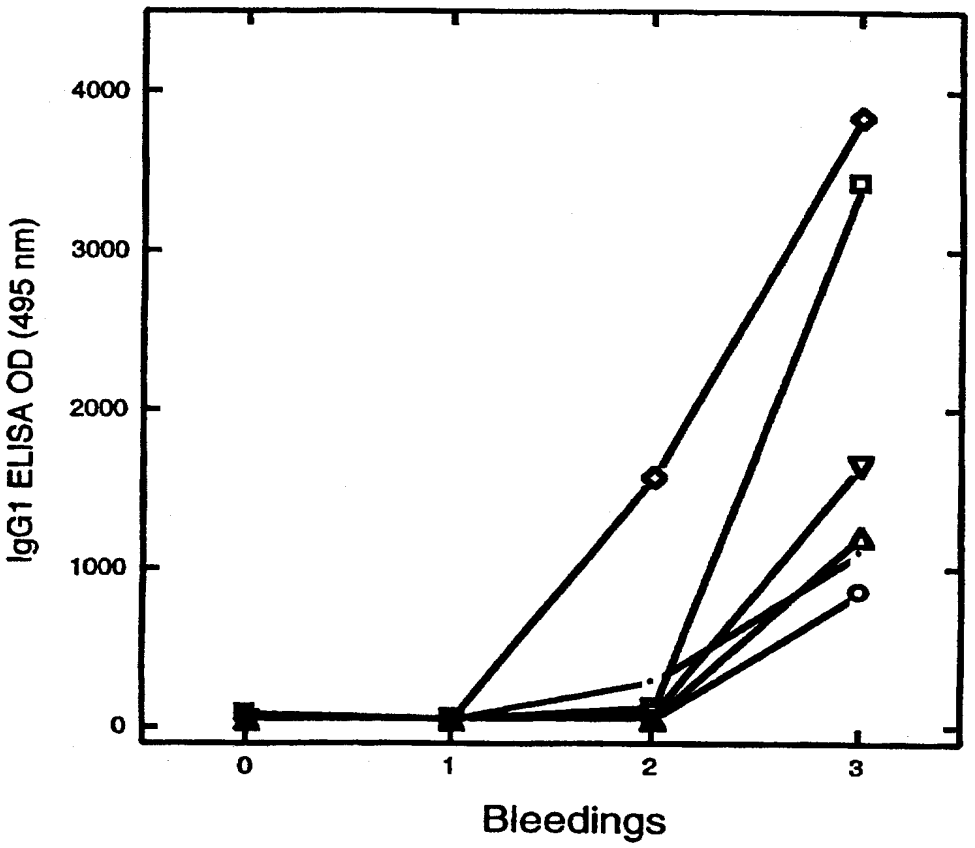
- Non-dendritic peptide carrier derivatized with L1 peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide - tuftsin s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + Freunds complete adjuvant
- Non-dendritic peptide carrier derivatized with L1 peptide i.p.
- Non-dendritic peptide carrier derivatized with L1 peptide - tuftsin i.p.

Fig. 27



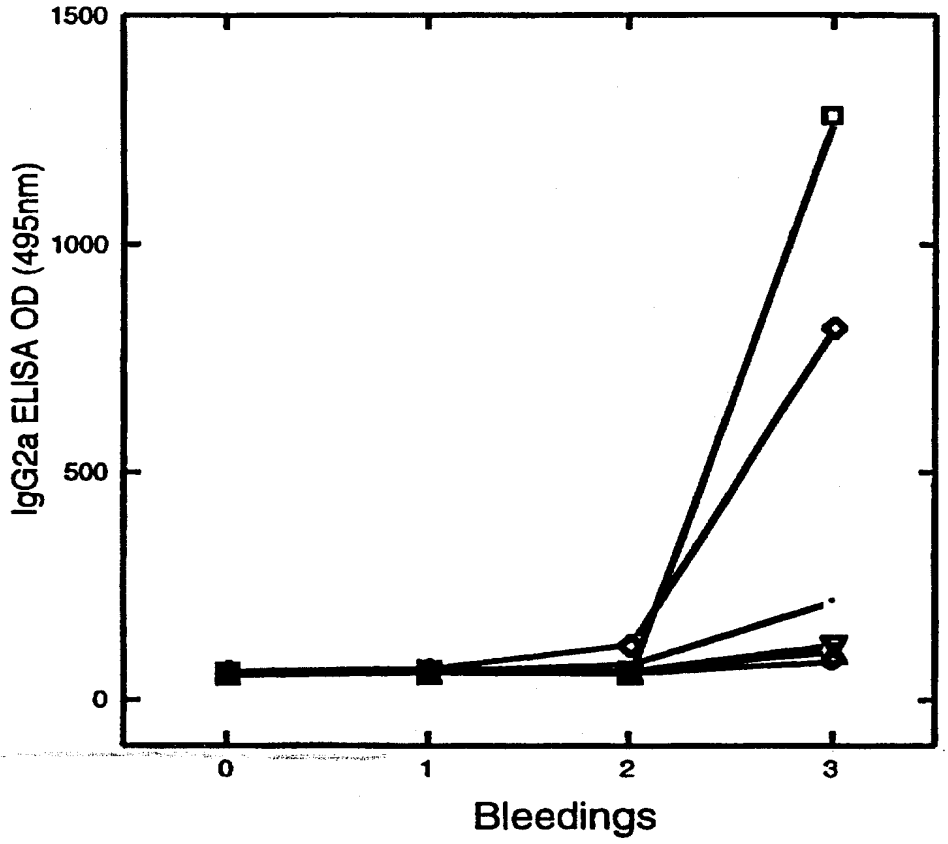
- Non-dendritic peptide carrier derivatized with L1 peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide - tuftsin s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + Freunds complete adjuvant s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide i.p.
- Non-dendritic peptide carrier derivatized with L1 peptide - tuftsin i.p.

Fig. 28



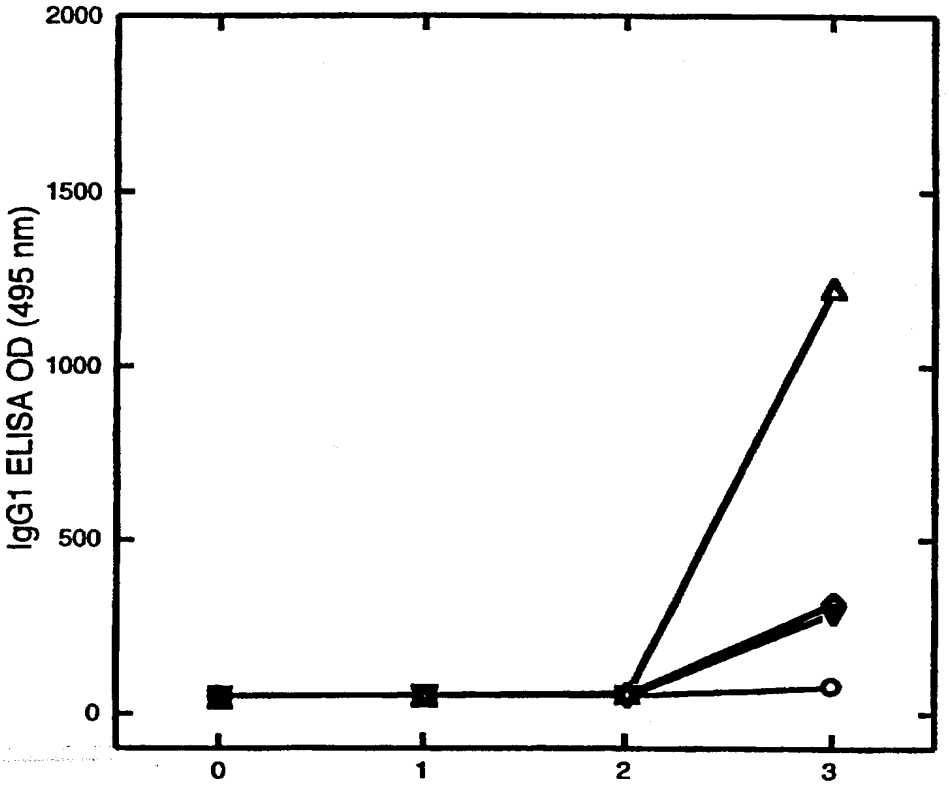
- Non-dendritic peptide carrier derivatized with L1 peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + Freunds adjuvant s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + rek. interferin-gamma s.c.
- Non-dendritic peptide carrier derivatized with L1 - IFN(1-39) peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 - IFN(95-133) peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 - IFN(1-39) + L1 - IFN(95-133) peptide s.c.

Fig. 29



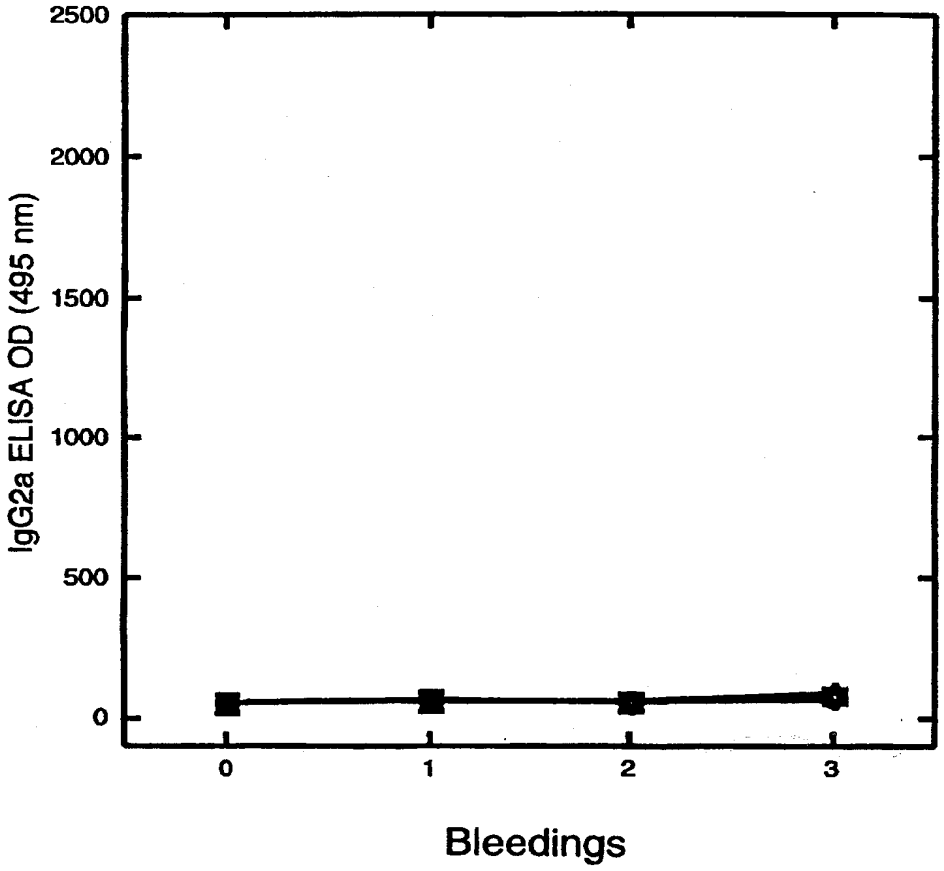
- ○ Non-dendritic peptide carrier derivatized with L1 peptide s.c.
- □ Non-dendritic peptide carrier derivatized with L1 peptide + Freunds adjuvant s.c.
- • Non-dendritic peptide carrier derivatized with L1 peptide + recombinant interferon-gamma s.c.
- ▽ Non-dendritic peptide carrier derivatized with L1 - IFN(1-39) peptide s.c.
- △ Non-dendritic peptide carrier derivatized with L1 - IFN(95-133) peptide s.c.
- ◇ Non-dendritic peptide carrier derivatized with L1 - IFN(1-39) + L1 - IFN(95-133) peptide s.c.

Fig. 30



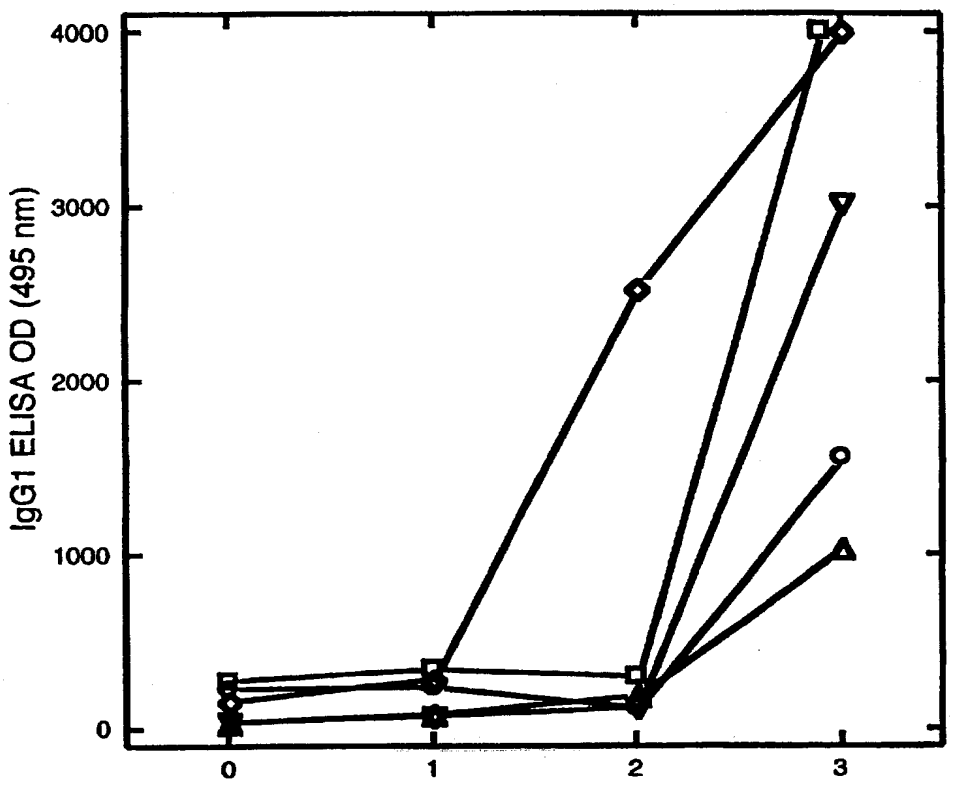
- Non-dendritic peptide carrier derivatized with L1 peptide i.p.
- Non-dendritic peptide carrier derivatized with L1 - IFN(1-39) peptide i.p.
- Non-dendritic peptide carrier derivatized with L1 - IFN(95-133) peptide i.p.
- Non-dendritic peptide carrier derivatized with L1 - IFN(1-39) + L1 - IFN(95-133) peptide i.p.

Fig. 31



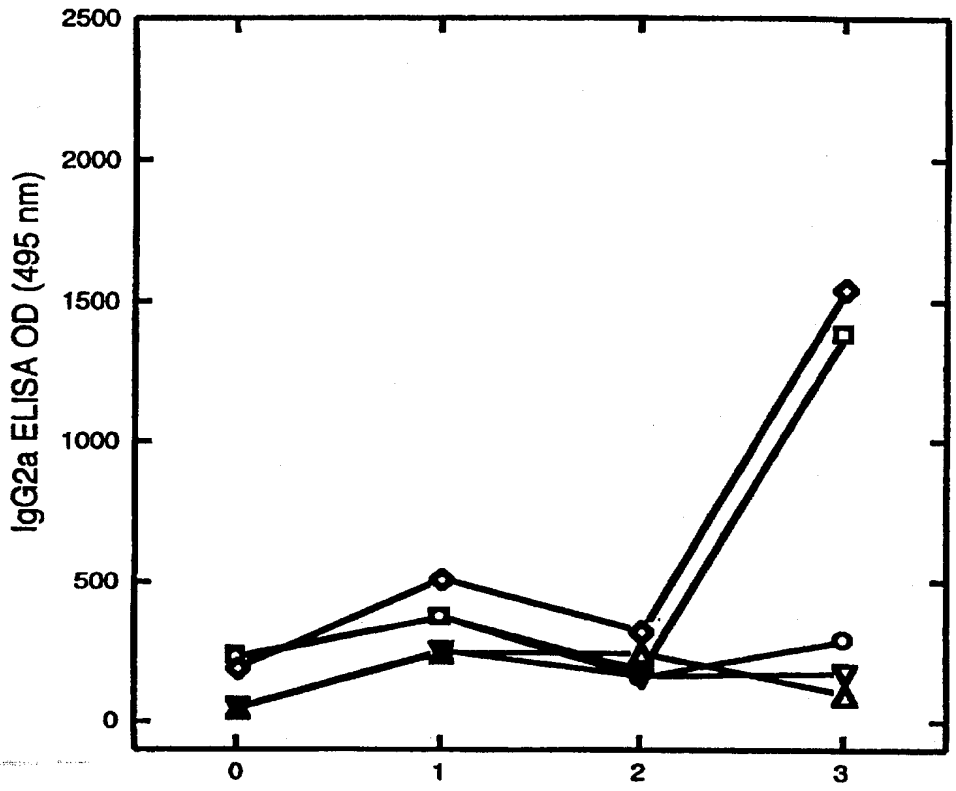
○ Non-dendritic peptide carrier derivatized with L1 peptide i.p.
▽ Non-dendritic peptide carrier derivatized with L1 - IFN(1-39) peptide i.p.
△ Non-dendritic peptide carrier derivatized with L1 - IFN(95-133) peptide i.p.
◇ Non-dendritic peptide carrier derivatized with L1 - IFN(1-39) + L1 - IFN(95-133) peptide i.p.

Fig. 32



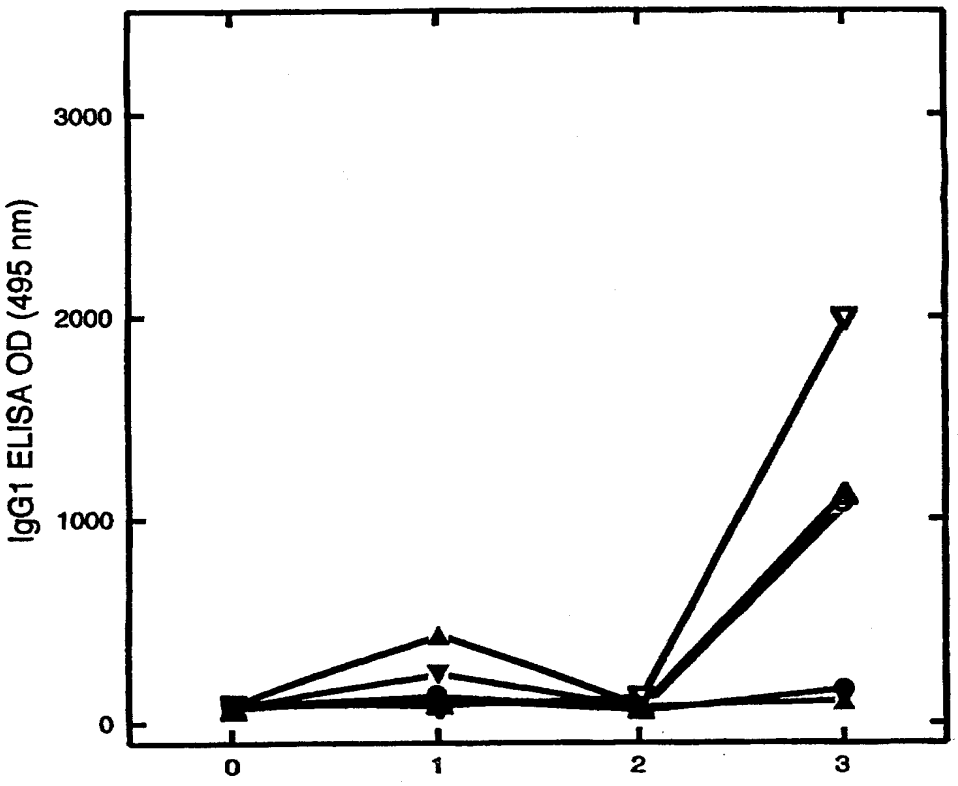
- Non-dendritic peptide carrier derivatized with L1 peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + Freunds complete adjuvant s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + recombinant TNF + alumn s.c.
- Non-dendritic peptide carrier derivatized with L1 - TNF(70-80) peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 - TNF(70-80) peptide i.p.

Fig. 33



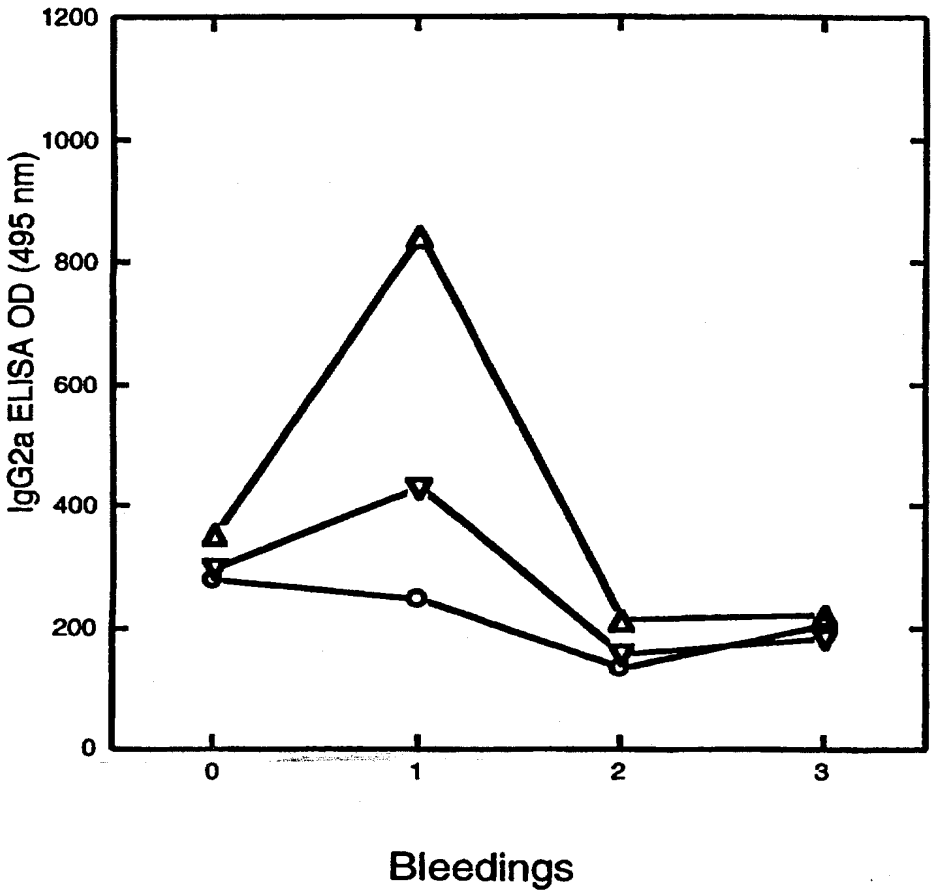
- Non-dendritic peptide carrier derivatized with L1 peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + Freunds complete adjuvant s.c.
- Non-dendritic peptide carrier derivatized with L1 peptide + recombinant TNF + alumn s.c.
- Non-dendritic peptide carrier derivatized with L1 - TNF(70-80) peptide s.c.
- Non-dendritic peptide carrier derivatized with L1 - TNF(70-80) peptide i.p.

Fig. 34



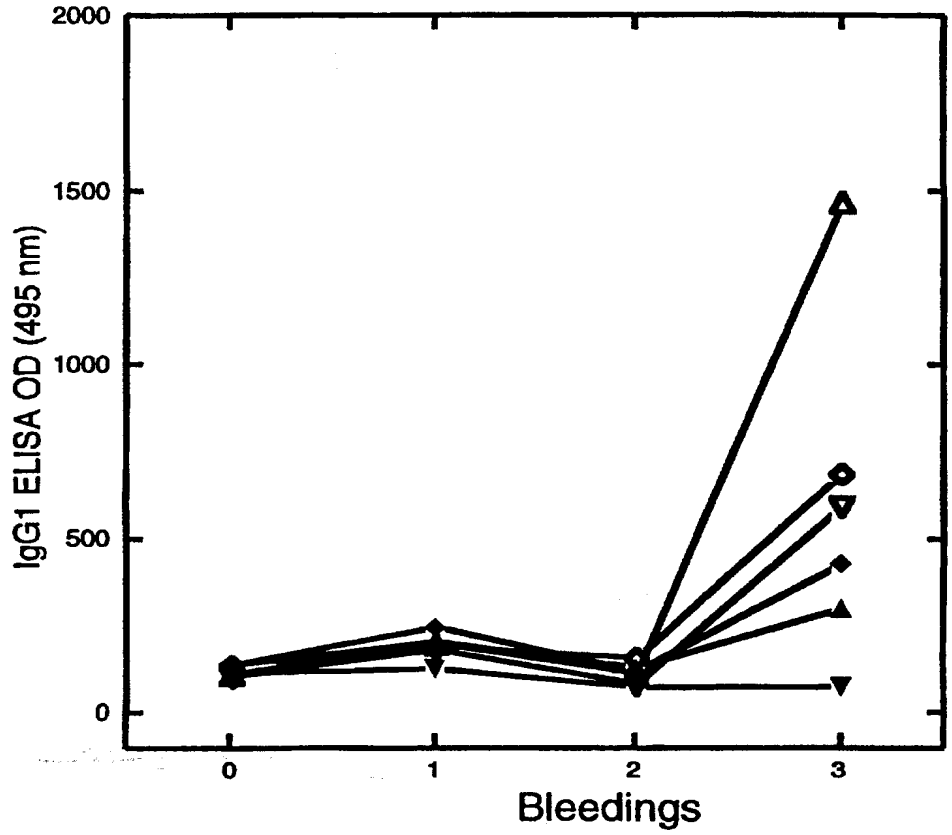
- Non-dendritic peptide carrier derivatized with L2 peptide s.c.
- Non-dendritic peptide carrier derivatized with L2 peptide i.p.
- Non-dendritic peptide carrier derivatized with L2 - TNF(70-80) peptide s.c.
- Non-dendritic peptide carrier derivatized with L2 - TNF(70-80) peptide i.p.
- Non-dendritic peptide carrier derivatized with L2 - tuftsin peptide s.c.
- Non-dendritic peptide carrier derivatized with L2 - tuftsin peptide i.p.

Fig. 35



○ Non-dendritic peptide carrier derivatized with L2 peptide i.p.
△ Non-dendritic peptide carrier derivatized with L2 - TNF(70-80) - branch i.p.
▽ Non-dendritic peptide carrier derivatized with L2 - tuftsin - branch i.p.

Fig. 36



◇ Non-dendritic peptide carrier derivatized with gp120 - IFN(1-39) + gp120 - IFN(95-133) peptide s.c.

◆ Non-dendritic peptide carrier derivatized with gp120 - IFN(1-39) + gp120 - IFN(95-133) peptide i.p.

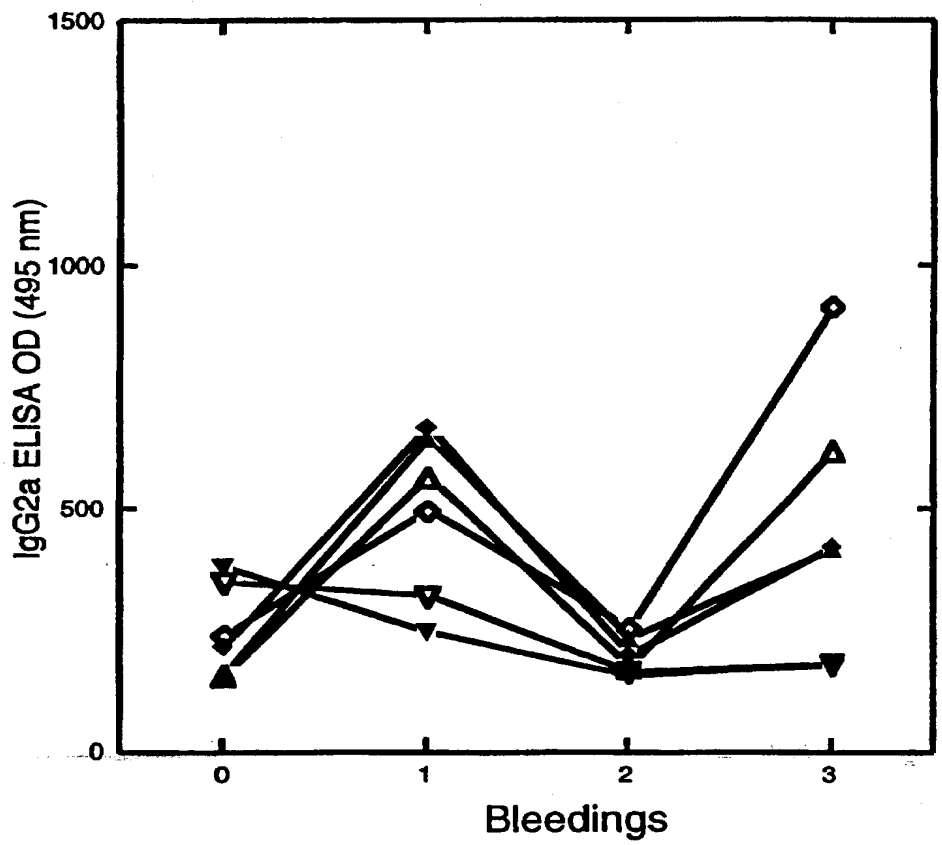
▽ Non-dendritic peptide carrier derivatized with gp120 tuftsin peptide s.c.

▼ Non-dendritic peptide carrier derivatized with gp120 - tuftsin peptide i.p.

△ Non-dendritic peptide carrier derivatized with gp120 - TNF(70-80) peptide s.c.

▲ Non-dendritic peptide carrier derivatized with gp120 - TNF(70-80) peptide i.p.

Fig. 37



◇ Non-dendritic peptide carrier derivatized with gp120 - IFN(1-39) + gp120 - IFN(95-133) peptides s.c.

◆ Non-dendritic peptide carrier derivatized with gp120 - IFN(1-39) + gp120 - IFN(95-133) peptides i.p.

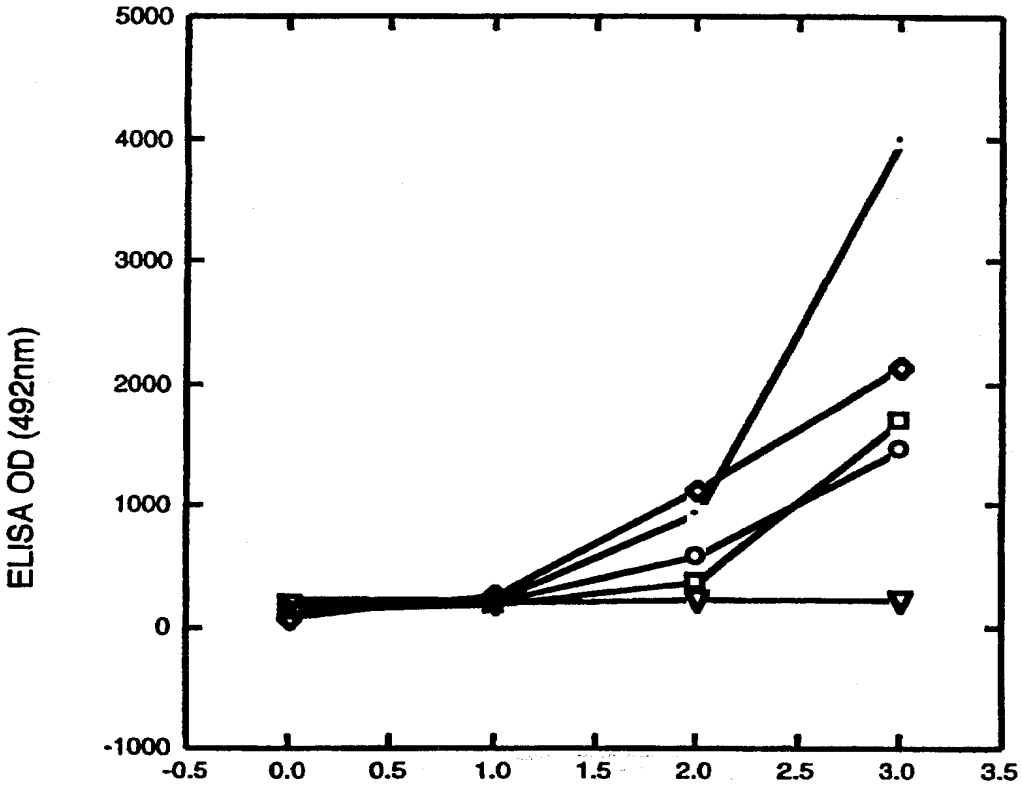
▽ Non-dendritic peptide carrier derivatized with gp120

▼ Non-dendritic peptide carrier derivatized with gp120 - tuftsin peptide i.p.

△ Non-dendritic peptide carrier derivatized with gp120 - TNF(70-80) peptide s.c.

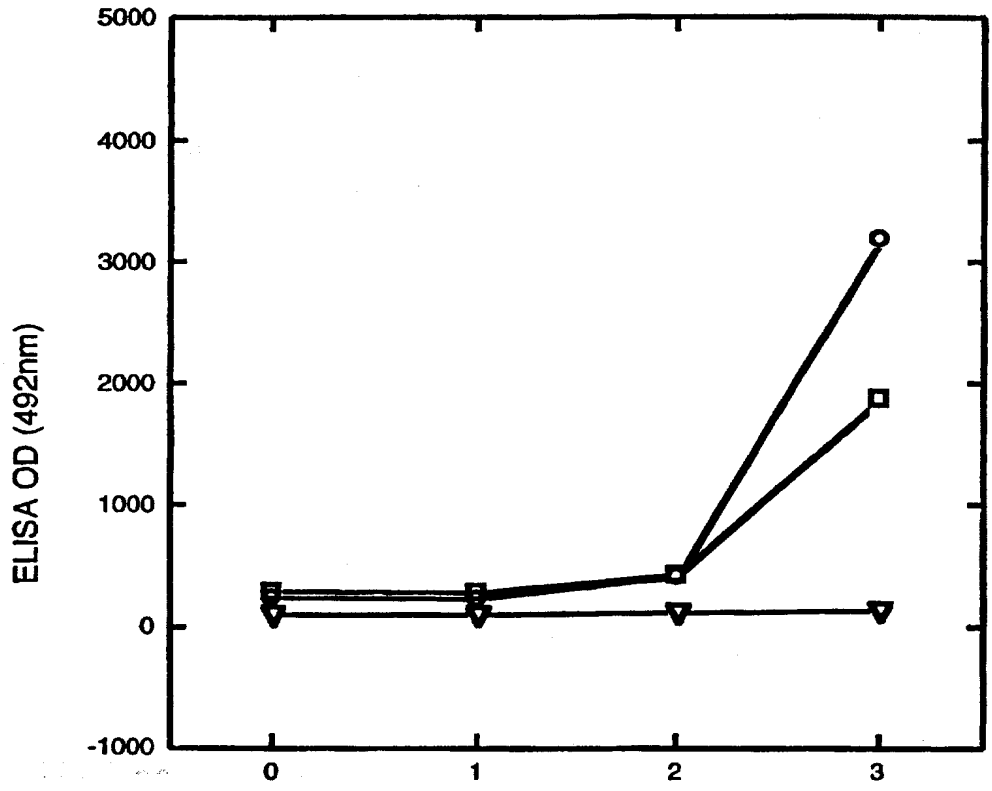
▲ Non-dendritic peptide carrier derivatized with gp120 - TNF(70-80) peptide i.p.

Fig. 38



- Non-dendritic peptide carrier derivatized with HIV-1 gp120 + IL-1 peptide
- Non-dendritic peptide carrier derivatized with HIV-1 gp120 peptide
- Non-dendritic peptide carrier derivatized with tuftsin control
- Non-dendritic peptide carrier derivatized with HIV-1 gp120 peptide + tuftsin
- Non-dendritic peptide carrier derivatized with HIV-1 gp120 peptide + Freunds adjuvant

Fig. 39



Non-dendritic peptide carrier derivatized with HIV-1 gp120 + IL-1 peptide

Non-dendritic peptide carrier derivatized with HIV-1 gp120 peptide

Non-dendritic peptide carrier control

Fig. 40

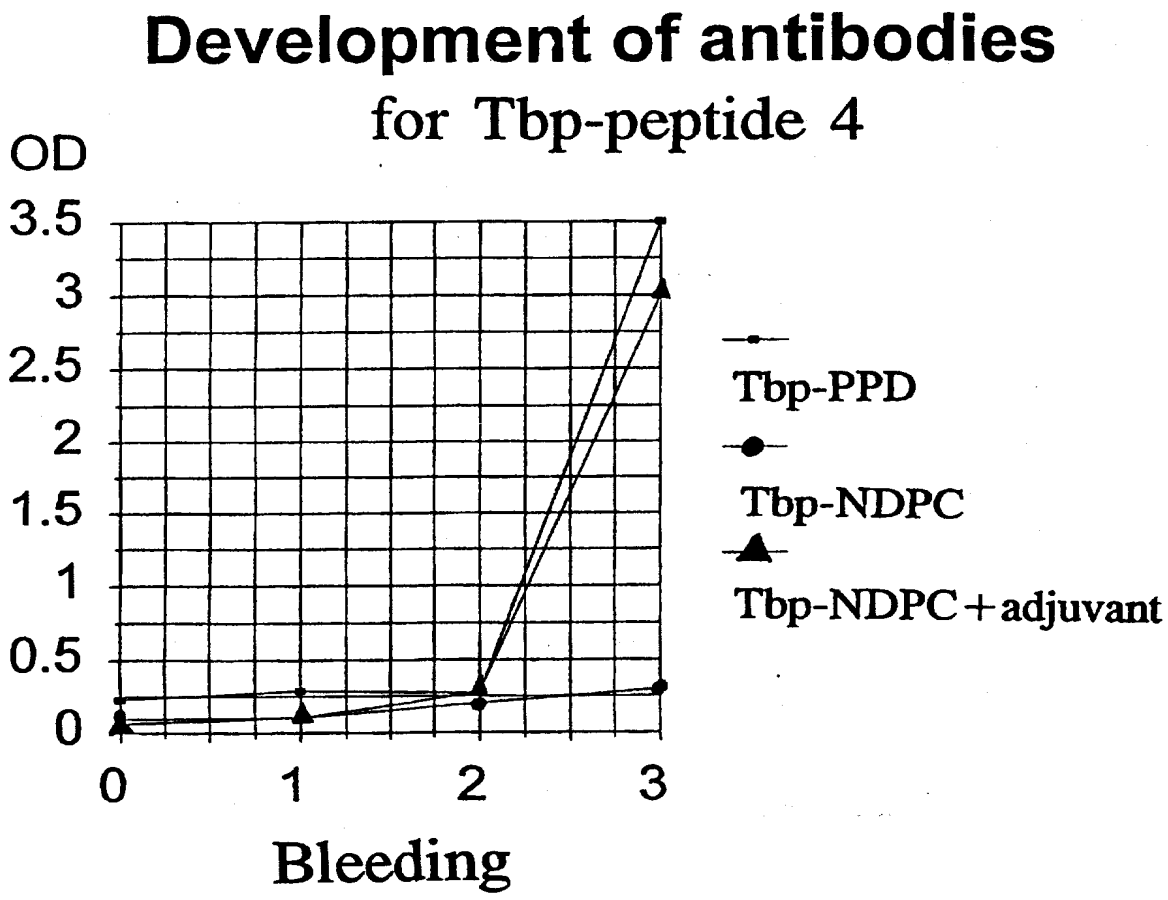

Development of antibodies
for Tbp-peptide 4
OD
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
Bleeding
Tbp-PPD
Tbp-NDPC
Tbp-NDPC+adjuvant

Fig. 41

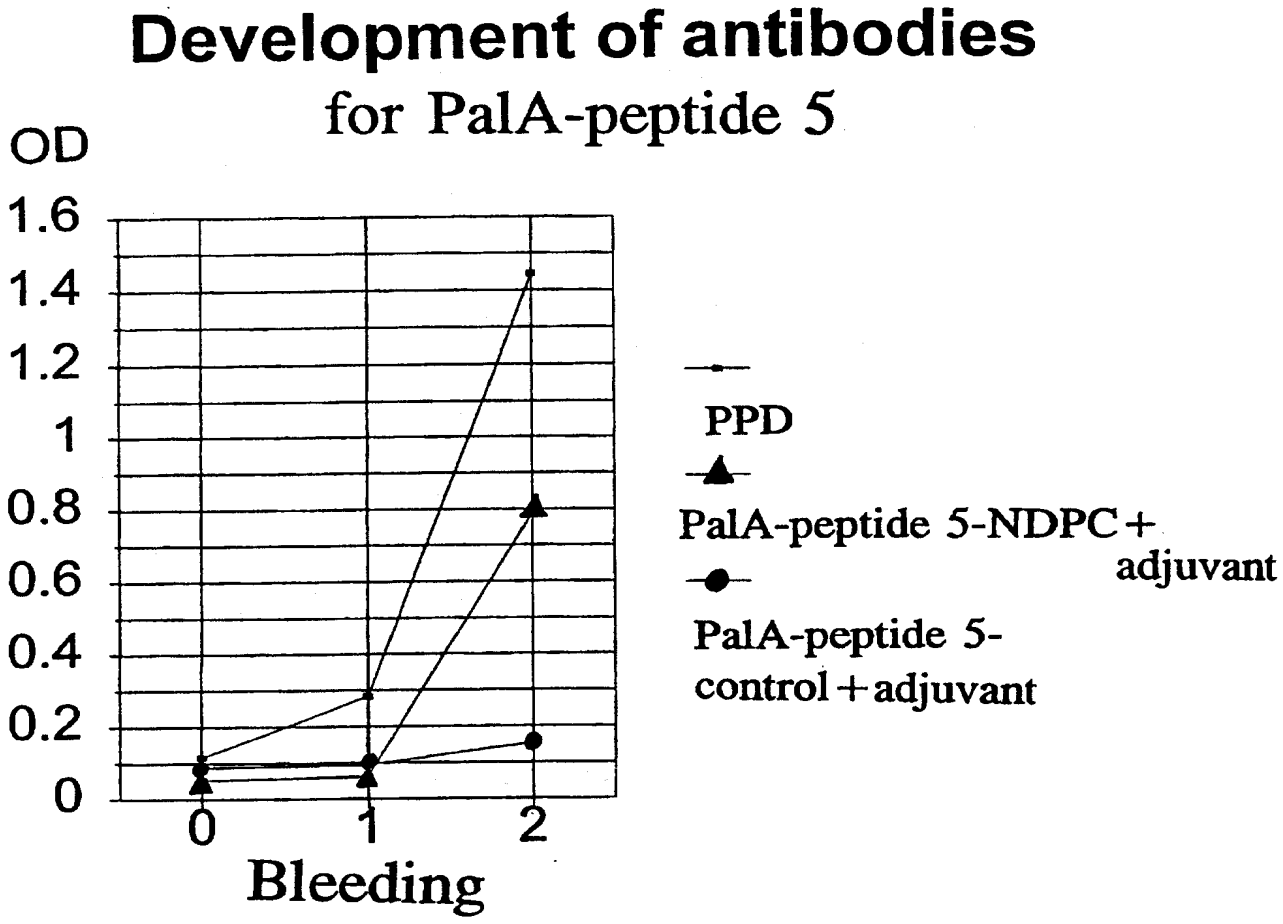

Development of antibodies
for PalA-peptide 5
OD
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
Bleeding
PPD
PalA-peptide 5-NDPC+ adjuvant
PalA-peptide 5-control+adjuvant

Fig. 42

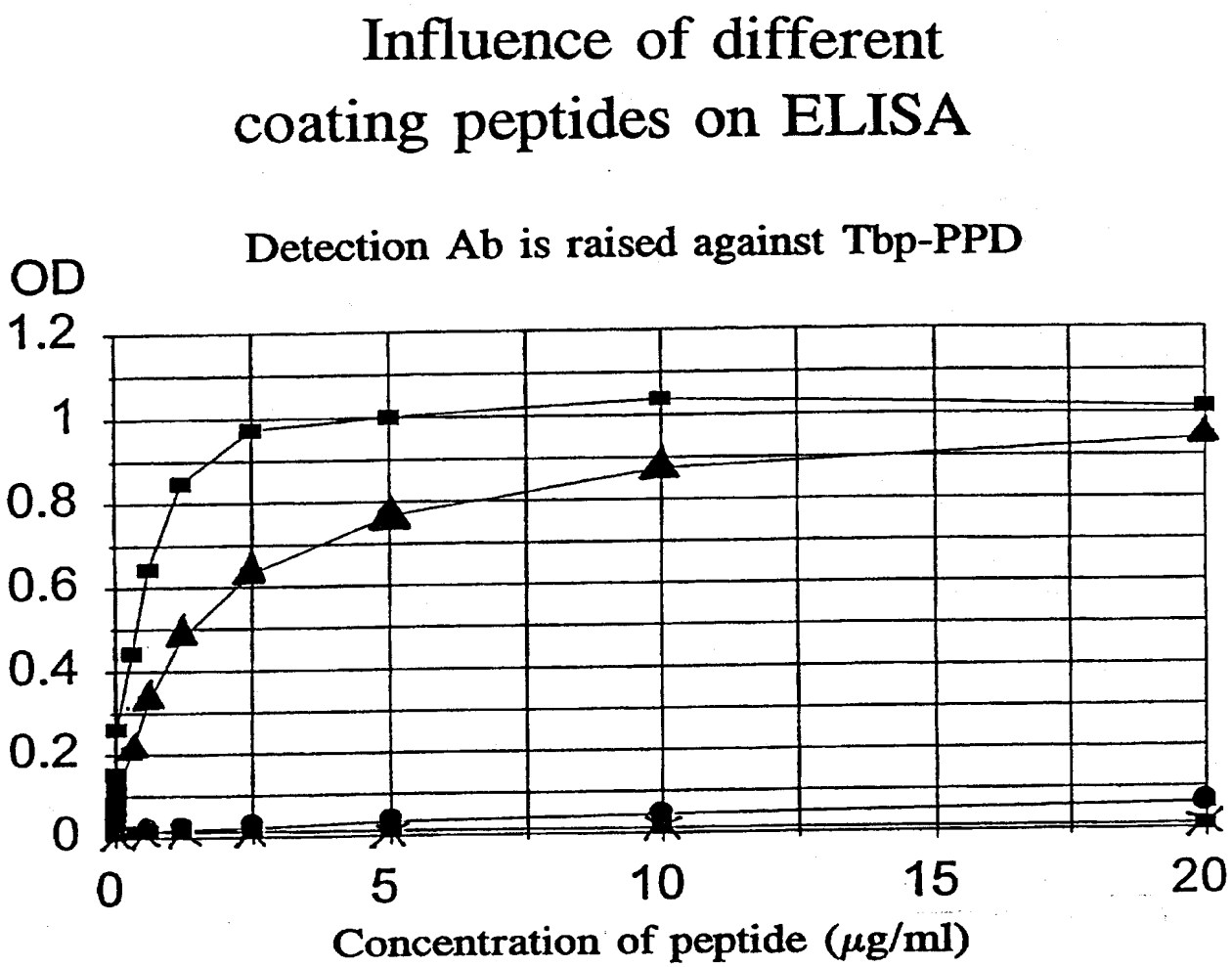

Influence of different coating peptides on ELISA
Detection Ab is raised against Tbp-PPD
OD
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
Concentration of peptide (µg/ml)
- Tbp-peptide 4 on structure 2 in ISCOM
- Tbp-peptide 4 on structure 3
- Palmitoylated Tbp-peptide 4 in ISCOM
- Palmitoylated Tbp-peptide 4

Fig. 43

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 97/00146

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/00 G01N33/68 A61K38/16 A61K39/385

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 31480 A (SYNTHETIC PEPTIDES INC ;HOUSTON MICHAEL E (CA); ZHOU NIAN E (CA);) 23 November 1995 cited in the application see page 4, line 1 - page 7, line 2 see page 21, line 35 - page 23, line 8; claims; examples ---	1-7, 9-12, 26-30, 43,46, 56-95
X	GB 2 282 813 A (MERCK & CO INC) 19 April 1995 cited in the application see page 7, line 5 - page 13, line 17; claims; examples --- -/--	1,8, 12-15, 56-95

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1997	07.08.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Fuhr, C

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 97/00146

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 04543 A (SCRIPPS RESEARCH INST ;DAWSON PHILIP E (US); KENT STEPHEN B H (US)) 16 February 1995 cited in the application see page 5, line 33 - page 8, line 2; claims; examples	1,8, 12-15, 56-95
X	WO 94 02506 A (SCHOOL OF PHARMACY UNIVERSITY ;TOTH ISTVAN (GB); GIBBONS WILLIAM A) 3 February 1994 see claims; examples	1,2,7,8, 12,13, 15, 20-22, 43-45, 56-95
A	WO 89 10348 A (SYNPHARM LTD ;SANTER VIVIEN BEDFORD (AU)) 2 November 1989 see the whole document	1-95
A	WO 95 33766 A (ONTARIO CANCER INST) 14 December 1995 see the whole document	1-95

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INTERNATIONAL SEARCH REPORT

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK 97/00146

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531480 A	23-11-95	AU 2441895 A	05-12-95
		CA 2190494 A	23-11-95
		EP 0759941 A	05-03-97
GB 2282813 A	19-04-95	NONE	
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		EP 0652896 A	17-05-95
WO 8910348 A	02-11-89	GB 2217319 A	25-10-89
		AU 3446189 A	24-11-89
		EP 0413709 A	27-02-91
		JP 4506203 T	29-10-92
WO 9533766 A	14-12-95	EP 0764170 A	26-03-97

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00146

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 53,54,66,68-70,80,81 and 93 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.